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Characterisation of the EIIA family protein in *Caulobacter crescentus*

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Faculté des Sciences

Characterisation of the EIIA family protein in *Caulobacter crescentus*

**Mémoire présenté pour l'obtention
du grade académique de master 120 en biochimie et biologie moléculaire et cellulaire**

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Janvier 2017

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Caractérisation de la famille de protéines EIIA chez *Caulobacter crescentus*

MARCHAND Elie

Résumé:

Les bactéries ont évolué afin de s'adapter à un environnement fluctuant, en ressentant chaque modification et en réagissant de manière appropriée à celle-ci. La production d'une alarmone, la guanosine hyperphosphorylée, appelée (p)ppGpp constitue un de ces mécanismes de réponse. Cette molécule de signal s'accumule lorsque la disponibilité des nutriments devient limitante. Une fois produit, le (p)ppGpp va modifier profondément le profil de transcription et de traduction de la cellule bactérienne afin de concentrer ses ressources pour faire face aux conditions de famine. Bien que le rôle du (p)ppGpp dans la réponse au stress nutritionnel est bien connu depuis des décennies, les mécanismes permettant la détection spécifique de carence en nutriments, et la régulation des taux de (p)ppGpp en accord avec cette carence spécifique, restent majoritairement incompris. Récemment, notre laboratoire a décrit le système phosphoenolpyruvate phosphotransférase lié à l'azote (PTS^{Ntr}) comme étant un mécanisme permettant l'accumulation de (p)ppGpp pendant un stress azoté chez *Caulobacter crescentus*. Il a été montré que trois composants du PTS^{Ntr} (EI^{Ntr}, Hpr and EIIA^{Ntr}) sont impliqués dans la régulation de SpoT, l'enzyme qui synthétise ou hydrolyse le (p)ppGpp. Plus spécifiquement, il a été découvert que la dernière protéine du phospho-relais -EI^{Ntr}- interagit avec SpoT afin d'inhiber spécifiquement son domaine hydrolase. Toutefois, un quatrième composant, appelé EIIA, et encodé par le gène ptsM, n'a encore jamais été étudié à ce jour.

Dans ce travail, nous avons caractérisé la régulation de la protéine EIIA^{Ntr} ainsi que le rôle potentiel joué par EIIA dans la réponse aux stress nutritionnels de *C. crescentus*. Finalement, nous avons aussi cherché de potentiels nouveaux régulateurs de SpoT grâce à la méthode du Tn-seq. Notre étude nous a permis d'identifier EIIA comme étant un régulateur de HPr et nous a également permis d'établir une liste de potentiels nouveaux activateurs et inhibiteurs de SpoT qui seront caractérisés dans le futur.

Mémoire de master 120 en biochimie et biologie moléculaire et cellulaire

Janvier 2018

Promoteur: Régis HALLEZ

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Characterisation of the EIIA family protein in *Caulobacter crescentus*

MARCHAND Elie

Abstract:

Bacteria evolved mechanisms to adapt to their fluctuating environments, by sensing any variations and to appropriately react to them. The production of an alarmone, the hyperphosphorylated guanosine called (p)ppGpp constitutes one of these mechanisms. This signalling molecule accumulates when nutrients become limiting. Once produced, (p)ppGpp will deeply modify the transcriptional and translational landscapes of bacterial cells in order to concentrate their resources to face starvation. Although the involvement of (p)ppGpp in bacterial stress response is well known for decades, mechanisms sensing specific nutrient starvation and accordingly regulating (p)ppGpp levels remains mostly unknown. Recently, our lab described the nitrogen-related phosphoenolpyruvate phosphotransferase system (PTS^{Ntr}) as one of these mechanism allowing (p)ppGpp accumulation during a nitrogen stress in *Caulobacter crescentus*. Three PTS^{Ntr} components (EI^{Ntr}, Hpr and EIIA^{Ntr}) have been shown to be involved in the regulation of the (p)ppGpp synthetase/hydrolase SpoT. In particular, it was found that the last protein of the phosphorelay – EIIA^{Ntr} – interacts with SpoT to specifically inhibit its hydrolase activity. However, a fourth component called EIIA and encoded by the *ptsM* gene was never studied so far.

In this work, we characterized the regulations of the EIIA^{Ntr} protein as well as the potential role played by EIIA in the nutrient stress response of *C. crescentus*. Finally, we also searched for potential new regulators SpoT by using a Tn-seq approach. Our study allowed to identify EIIA as a regulator of HPr as well as to list potential new activators and inhibitors of SpoT that should be further characterized in the future.

Mémoire de master 120 en biochimie et biologie moléculaire et cellulaire

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Promoteur: Régis HALLEZ

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Introduction

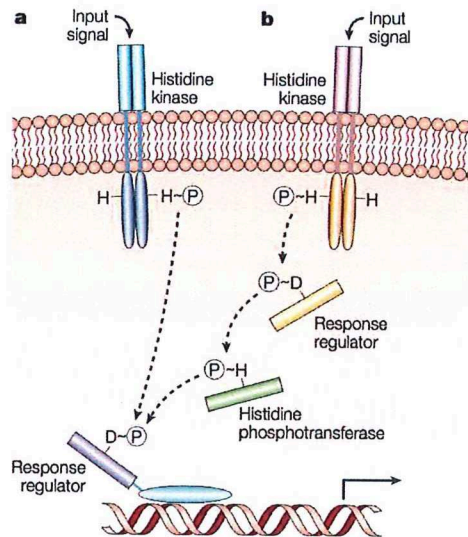


Figure1: Two component system

The two components systems are a histidine kinase, that respond to an input signal and phosphorylates a response regulator. The response regulator is usually a transcription factors **(a)**. Multicomponent phosphorelays also exist **(b)**.

From Skerker et al. 2004

I. INTRODUCTION

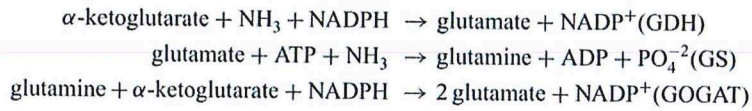
A. How do bacteria sense and respond to environmental changes?

In their environment, bacteria have often to face fluctuating environments and adapt to them notably by modulating gene expression. There are at least three regulatory systems involved in the adaptative response to nutrients limitation. The first is the two-component signal transduction family, usually composed of a histidine kinase (HK) and a response regulator (RR)[1]. Most of the time, the HK is a transmembrane protein that can sense extracellular changes and, in response to a stimulus, phosphorylates its cognate RR. As in most cases, the RR is a transcription factor, the phosphorylated RR will express a set of genes necessary to adequately respond to the starvation. The second system is the phosphoenolpyruvate:phosphotransferase system (PTS) [2]. The canonical PTS is a phosphorelay involved in nutrient uptake, composed of 3 general proteins (EI, HPr, EIIA) and specific components (EIIB and EIIC), each pair of EIIBC allowing transport of specific carbohydrates (glucose, mannose, N-acetyl glucosamine, ...). There is also a nitrogen-related PTS (PTS^{Ntr}), only composed of 3 proteins (EI^{Ntr}, NPr and EIIA^{Ntr}). In contrast to PTS, PTS^{Ntr} carries out only regulatory functions in response to nutrient starvation. The last system is based on small secondary messengers such as the guanosine tetra-/penta-phosphate, referred to as (p)ppGpp, [3] which has been linked to stress response in bacteria, especially upon nutrient starvation. Also called alarmone, (p)ppGpp modulates a wide range of essential processes such as transcription, translation and cell cycle progression but also motility, biofilm formation, persistence to antibiotics, virulence, In the following lines, we will describe these three regulatory systems and more specifically the hybrid PTS^{Ntr}/PTS system of *Caulobacter crescentus*.

1. Two-component systems (TCS)

Two-component systems have been shown to mediate a large number of bacterial adaptative responses [4]. Classical two-component systems are composed of a HK and a RR. Membrane-associated HK is composed of 3 parts: (i) an extracytoplasmic domain that acts as a sensor of external changes, (ii) transmembrane domains allowing the anchoring of the HK to the inner membrane and (iii) an intracellular histidine kinase domain. When an extracytoplasmic signal activates the HK, it undergoes an auto-phosphorylation on a conserved histidine (figure 1a). The activated HK then transfers its phosphoryl group to a conserved aspartate of its cognate RR. Once activated by phosphorylation, the RR that harbors a DNA binding domain acts as a transcription factor by regulating transcription of the genes whose products are required to respond to the external stimuli (figure 1) [4], [5].

A.



B.

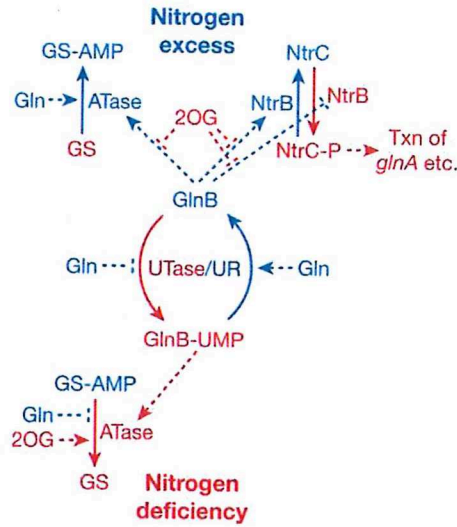


Figure2:

A. Reaction of synthesis of Glutamine and Glutamate in *E.coli*.

B. Regulation of Glutamine Synthetase (GS) under nitrogen excess or nitrogen deficiency

From Leigh et al. 2007

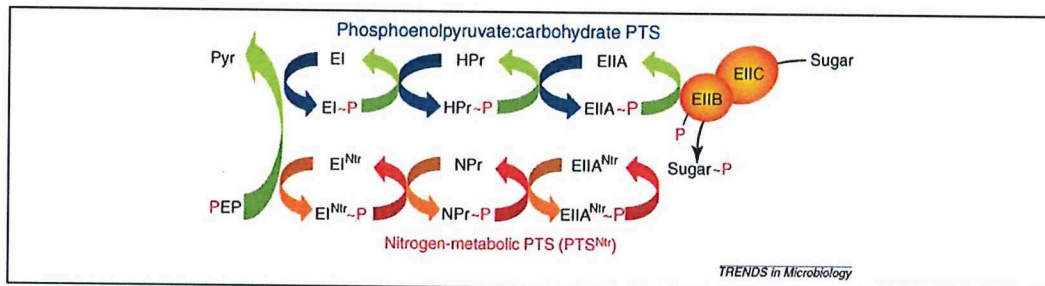


Figure 3: Phosphotransferase systems in bacteria

The phosphoenolpyruvate:carbohydrates PTS (above), associated with permeases, is activated by PEP and permit the sugar intake.

The nitrogen metabolic PTS, is also activated by the PEP but it is described to have rather regulatory functions.

From Pflüger-Grau et al. 2010

A more complex phosphorelay involves more than 2 components, with a Histidine Phosphotransferase (HPT) relaying the phosphoryl group from a RR domain to another one (figure 1b).

NtrBC is a canonical TCS used by many bacteria to respond to nitrogen starvation. Nitrogen is an essential nutrient for all living cells since it is involved in the building of essential macromolecules (amino acids and nucleic acids). Bacteria are able to assimilate various nitrogen sources but all of them will be converted into ammonium [6]. Ammonium can be assimilated into glutamate (α -ketoglutarate + NH_4^+ + NADPH + H^+ \leftrightarrow glutamate + NADP⁺) by the NADP-dependent glutamate dehydrogenase (NADP-GDH) or into glutamine (glutamine + NH_4^+ + ATP \leftrightarrow glutamine + ADP + Pi) by the glutamine synthase (GS). Both glutamate and glutamine will then be able to give the amino group for biosynthesis [7]. Glutamine is the major nitrogen donor in *E. coli* and also acts as a nitrogen sensor [6], [8]. Under nitrogen stress, the nitrogen uptake is activated (red in figure 2). In such conditions, the GlnB regulator will be uridylylated (+UMP) by GlnD, which is the universal sensor for nitrogen in bacteria. As a consequence, GlnB::UMP (i) will stimulate removing of adenylyl (AMP) groups from the GS to activate it and (ii), at the same time, will stop stimulating the phosphatase activity of NtrB, which will increase the phosphorylation level of NtrC. Since NtrC~P activates transcription of *glnA* coding for the GS, both the levels and the activity of the GS will increase to palliate the nitrogen starvation. Conversely, nitrogen excess will generate high intracellular concentration of glutamine that will inhibit the uridylyltransferase activity of GlnD and stimulate removing of UMP groups from GlnB. In this configuration, GlnB will not only stimulate the dephosphorylation of NtrC~P, and thus inhibit expression of *glnA*, but also favor inhibition of the GS activity by adenylylation (blue in figure 2) [7].

B. Phosphoenolpyruvate phosphotransferase systems (PTS)

The PTS is a second mechanism used by bacteria to sense and respond to environmental variations such as nutrient fluctuations. It has been shown to be involved in the uptake and metabolism of carbon, nitrogen and phosphate metabolisms [9], [10]. Two different PTS coexist in *E. coli*, the canonical one (PTS) involved in carbohydrates uptake and the regulatory one (PTS^{Ntr}) [11], [12](figure 3). Both systems are phosphorelays initiated by phosphoenolpyruvate (PEP).

1. Components of the canonical PTS

The first protein of PTS is called EI and is encoded by *ptsI*. It contains two domains: a C-terminal domain required for dimerization, regulation and interaction with PEP and a N-terminal domain important for binding HPr and harboring a conserved histidine residue used as the phosphorylation site. Dimerization of EI is required for its autophosphorylation mediated by PEP and EI~P phosphorylates reversibly HPr [12], [13].

HPr, the second protein of the PTS pathway is encoded by *ptsH* and is highly conserved among bacteria. HPr is phosphorylated by EI also on a conserved histidine residue [12]. HPr can also be phosphorylated by a kinase, HPrK, on a serine residue [14]. Depending on the metabolic state, the serine residue will be more or less phosphorylated. For example, high level of ATP and fructose-1,6-bisphosphate induced by a rapid metabolism of the carbon source stimulates the HprK-dependent phosphorylation of HPr. In contrast, high levels of inorganic phosphate favors the unphosphorylated serine state of HPr [14]. Basically, in *Bacillus subtilis*, the phosphorylation of the serine residue of HPr (HPr^{Ser~P}) strongly inhibits the phosphotransfer on histidine residue between EI and HPr (HPr^{His~P}), which lowers the uptake and phosphorylation of the carbohydrate. HPr^{Ser~P} is also able to interact with the transcription factor CcpA, a catabolite activator in *B. subtilis* [15], [16]. In *E. coli* and most proteobacteria, HprK is missing but the serine is conserved and more interestingly, the replacement of this conserved serine residue by an aspartate residue mimics the effects induced upon phosphorylation of HPr by HPrK (HPr^{Ser~P}) [17] (Figure 4A).

The final effectors of the canonical PTS in *E. coli* are EII proteins (A, B, C), making complexes that form a transmembrane cluster that mediates the sugar uptake and phosphorylation. EIIA domains receive the phosphoryl group from HPr on a conserved histidine residue. The phosphoryl group is then transferred onto EIIB that will transfer the phosphoryl group on the carbohydrate thanks to EIIC during the uptake of the sugar through EIIC [18]. In *E. coli*, and in absence of carbohydrate, EIIA cannot get rid of its phosphoryl group. Phosphorylated EIIA (EIIA~P) will thus interact and activate the adenylate cyclase (AC). Once activated, AC will convert ATP to cyclic AMP (cAMP), a second messenger able to bind the transcriptional activator CRP. Then cAMP-CRP complexes will activate expression of catabolic enzymes in response to a low carbohydrate uptake. In contrast, unphosphorylated EIIA proteins bind and inactivate catabolic enzymes and carbohydrate transporters [18] (Figure 4B).

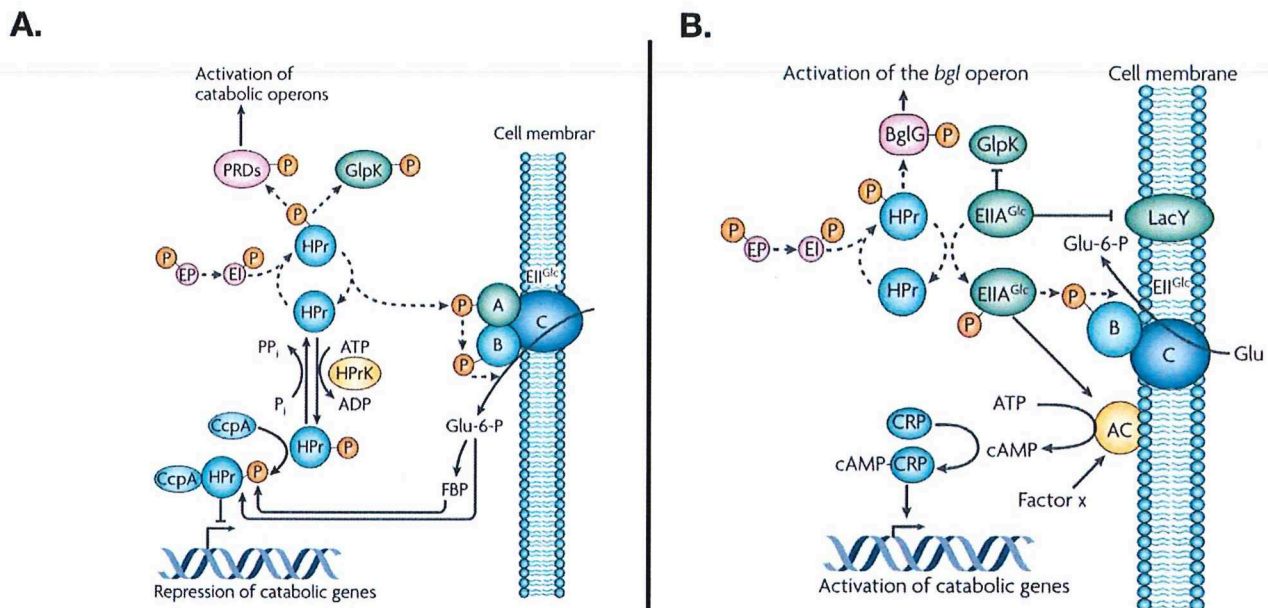


Figure 4: PTS in *B. subtilis* and *E. coli*

- A.** The canonical PTS in *B. subtilis*, phosphorylation of HPr by HPrK, leading to activation of HPr as transcription factor
- B.** The canonical PTS in *E. coli*, associated with carbohydrate intake permeases, is activated by PEP. When no sugar, EIIA~P activates adenylate cyclase, that activates transcription factors.

From Görke et al. 2008

2. The nitrogen-related PTS (PTS^{Ntr})

The PTS^{Ntr} was first described in *E. coli* and seems to carry out only regulatory functions. The PTS^{Ntr} is composed of similar components than those of canonical PTS (EI^{Ntr}, NPr and EIIA^{Ntr}) but without EIIB and EIIC. The first reason why PTS^{Ntr} was associated with nitrogen metabolism came from the syntenicity between *rpoN*, *ptsN* and *ptsO*. Indeed, in *E. coli* *ptsN* encodes the sigma factor 54 (σ_{54}) that recruits the RNA polymerase to promoters driving transcription of genes involved in nitrogen uptake and metabolism. The *rpoN* operon includes *rpoN* but also *yhbH*, *ptsN*, *yhbJ* and *ptsO*. Since *ptsN* and *ptsO* code respectively for EIIA^{Ntr} and Npr (a HPr homolog), their co-expression suggested PTS^{Ntr} played a role in nitrogen stress response. It is noteworthy that EI^{Ntr} protein is encoded by *ptsP* located separately on the chromosome [11]. In contrast to the sugar PTS, the PTS^{Ntr} system is mostly absent from gram-positive bacteria.

EI^{Ntr} is similar to EI but contains a GAF domain at its N-terminal extremity. GAF, standing for cGMP phosphodiesterases, *Anabaena* adenylate cyclases and *Escherichia coli* EhIA, are domains involved in signal perception [11]. NPr is a phosphate carrier transferring the phosphoryl group from EI^{Ntr} to EIIA^{Ntr}.

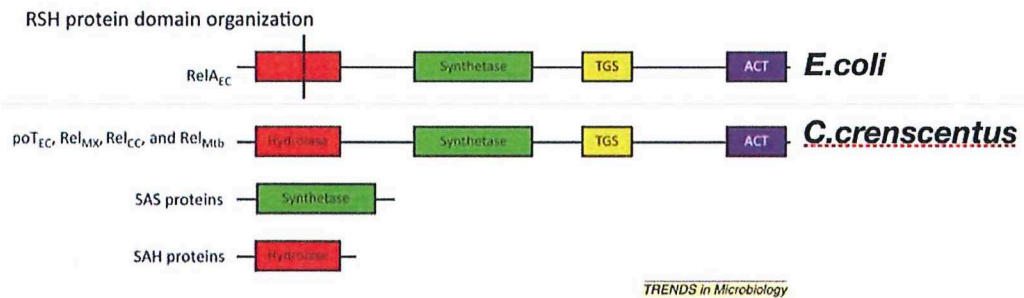


Figure 6: RelA/SpoT schematic representation in *E. coli* and *C. crescentus*

Schematic representation of *E. coli* RelA, SpoT from *C. crescentus*, and SAS, SAH proteins. SpoT is a bifunctional enzyme presenting a hydrolase and a synthetase domain, while RelA has a synthetase domain and a non functional hydrolase domain.

groups of RSH proteins: (i) the *bona fide* long bifunctional HD/SD called Rel or SpoT, (ii) the long monofunctional SD called RelA, (iii) the short mono-domain synthetase called SAS and (iv) the short mono-domain hydrolase called SAH. SAS and SAH stand for Small Alarmone Synthetase or Hydrolase, respectively. Whereas gram-negative bacteria have only long RSH, gram-positive bacteria have both long and short RSH.

The C-terminal part of the long RSH is composed of regulatory domains called TGS (ThrS, GTPase and SpoT) and ACT (Aspartokinase, Chorismate and TyrA). The exact function of the C-terminal domains remains unclear but it seems these domain regulate activity of the N-terminal catalytic domains [26]–[28].

In *E. coli* two RSH coexists: RelA and SpoT (Figure 6). SpoT is a bifunctional HD/SD protein with a strong HD activity and much less pronounced SD activity than the one of RelA. Nevertheless, the SD activity of SpoT was shown to be activated upon several stress conditions (fatty acids starvation, carbon starvation phosphate starvation, ...) [3], [18], [29]. The second RSH - RelA - has a strong SD activity but an inactive hydrolase domain that makes SpoT essential as being the only protein able to degrade (p)ppGpp produced by RelA. In contrast to SpoT, the SD activity of RelA is induced upon amino acid deprivation and requires the binding of RelA to ribosomes. Under amino acids scarcity, deacylated tRNA will accumulate into the A-site of the ribosome, stimulating a conformational change of RelA on the ribosome. Indeed, RelA will adopt an “open” conformation that facilitates (p)ppGpp synthesis [28], [30], [31]. This presence of uncharged tRNA into the ribosome seems to also inhibit the HD activity of SpoT [32]. As a consequence, (p)ppGpp levels quickly raise. Fatty acid starvation is the only known

activating signal of the of SpoT_SD activity in *E. coli* for which a molecular mechanism has been discovered [33].

2. Targets and functions of (p)ppGpp

As mentioned above, (p)ppGpp induces a systemic change in response to nutrients starvation and a few (p)ppGpp targets have been identified. DNA replication is one of the cellular process targeted by (p)ppGpp. In the gram-positive bacterium *B. subtilis*, it has been shown that (p)ppGpp binds DnaG to inhibit its activity [24]. DnaG is a DNA primase that synthesises a RNA primer necessary to allow DNA replication to proceed [34].

The alarmone can also interfere with transcription by different ways, either by directly interfering with some transcripts or by binding the RNA polymerase (RNAP) [24]. Two different (p)ppGpp binding sites have been identified on the RNAP of *E. coli* [35]–[37]. In the gram-positive *B. subtilis*, (p)ppGpp interferes with transcription by limiting GTP synthesis, since GTP is a nucleotide often used to initiate transcription at the +1 site.

The translation constitutes another cellular process targeted by (p)ppGpp. As GDP/GTP and (p)ppGpp are highly similar molecules, which differs only by their pyrophosphate moiety, their similarities have been shown to impact ribosome assembly and translation at the initiation, elongation and termination steps [38]. In fact, those translation steps are regulated by GTPases for which (p)ppGpp can easily compete with GDP or GTP.

D. *Caulobacter crescentus*

1. A bacterial model to study cell cycle and stress response

Caulobacter crescentus became a bacterial model to study cell cycle regulation essentially because it divides asymmetrically. *C. crescentus* is a gram-negative oligotrophic α -proteobacterium living in all aquatic environments, and thus facing regular nutrient deprivation [5], [39], [40]. The asymmetric cell division of *C. crescentus* gives birth to two morphologically and functionally different daughter cells: a large stalked cell anchored to the substrate thanks to an efficient glue, called holdfast and present at the tip of the stalk, and a small swarmer cell able to escape from starvation conditions (figure 7) [41], [42].

The two daughter cells initiate different developmental programs. The stalked cell is not motile but able to initiate DNA replication [43]. It is important to note that unlike lots of bacteria, *C. crescentus* replicates its chromosome only once per cell cycle. The swarmer cell harbors a single polar flagellum, pili and a chemotaxis apparatus. In contrast to the stalked cell, the swarmer cell cannot initiate DNA replication but rather enters into a pre-

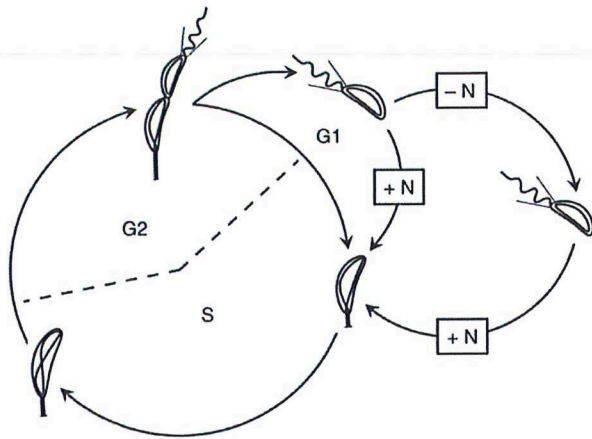


Figure 7: Asymmetric division of *C. crescentus*

Under nitrogen deprivation, the bacteria extend the G1, non-replicative phase, until stress is removed by addition of nitrogen.

After Ronneau et al. 2016

replicative G1 phase. Under nutrient rich conditions, the swarmer cell will rapidly leave the G1 phase to start a DNA replication cycle and, concomitantly, differentiate into a stalked cell. Interestingly, when some nutrients become limiting, the G1 phase of the swarmer cell will be elongated, thereby delaying differentiation and entry into the cell cycle [10], [44], [45]. This mechanism has been interpreted as a stress response allowing *Caulobacter* swarmer cells to flee far from scarcity and to colonize better environments. The delay in the G1-S transition has been linked to nitrogen and carbon starvation, two conditions known to lead to (p)ppGpp accumulation [46]–[48]]. More recently, the hybrid PTS has been shown (i) to sense nitrogen availability and in response to it, (ii) to regulate the activity of the unique bifunctional HD/SD protein called SpoT [44]. It is important to note that the mechanism by which (p)ppGpp interferes with cell cycle progression is not yet understood.

2. PTS in *Caulobacter crescentus*

In *C. crescentus*, (p)ppGpp abundance is dictated by the unique (p)ppGpp synthetase/hydrolase SpoT protein (Figure 6). Only nitrogen or carbon starvation can induce (p)ppGpp accumulation. Recently, our lab found that the PTS system was critical for *Caulobacter* to respond to a nitrogen starvation by modulating SpoT activities [44].

C. crescentus harbours 3 different PTS (Figure 8), two paralogous systems involved in uptake of N-acetyl glucosamine (NAG) encoded in two separate operons, and one hybrid system composed of 2 nitrogen-specific components (EI^{Ntr} and EII^{Ntr}) and 3 sugar specific ones (HPr, EIIA and HPrK). The first protein, EI^{Ntr} encoded by *ptsP* harbors a GAF domain described to bind glutamine and a PEP-phosphotransferase domain stimulating autophosphorylation of EI^{Ntr} by PEP [49]. Once autophosphorylated, EI^{Ntr} will transfer its phosphoryl group to the histidine 18 of HPr (encoded by *ptsH*), which in turn will

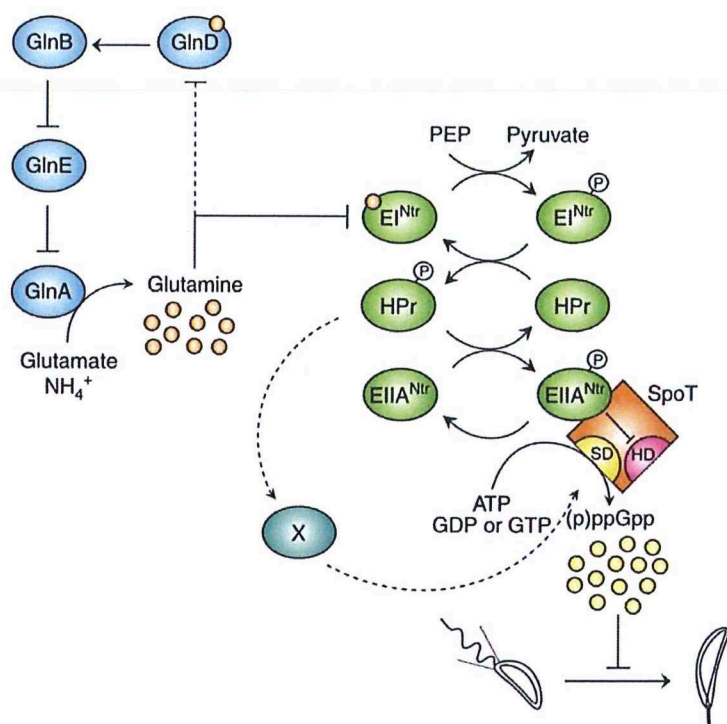


Figure 8: PTS^{Ntr} mediated accumulation of (p)ppGpp mediated in response of glutamine pool.

When abundant, glutamine inhibits the phosphoryl transfer from PEP to EI^{Ntr} . Under nitrogen starvation, PTS^{Ntr} is activated leading to the (p)ppGpp mediated stress response.

After Ronneau et al. 2016

phosphorylate the histidine 66 of EIIA^{Ntr} (encoded by *ptsM*) [44]. Once phosphorylated, EIIA^{Ntr}~P has been shown to inhibit the hydrolase activity of SpoT by interacting with the ACT domain (S. Ronneau et al. unpublished). As the autophosphorylation of EI^{Ntr} is inhibited by glutamine binding to the GAF domain, all the components of the PTS are highly phosphorylated upon nitrogen starvation. Indeed, in such conditions, the glutamine pool drops quickly, which relieves EI^{Ntr} inhibition. HPrK (encoded by *hprK*) is similar to the HPr kinase/phosphorylase of *B. subtilis* and EIIA (encoded by *ptsM*) is similar to the sugar-specific EIIA protein of *E. coli*. The role of these two proteins in stress response of *C. crescentus* is currently unknown.

Objectives

II. Objectives

PTS^{Ntr} has been shown in several bacteria to play a critical role in stress response. *C. crescentus* harbours two EIIA-like proteins, but only one of them (EIIA^{Ntr}) has been shown to regulate the response to nutrients starvation, notably by inhibiting the hydrolase activity of SpoT. In contrast, the second EIIA protein remains uncharacterised but preliminary unpublished data in the lab suggest EIIA could also regulate SpoT activity.

During this Master thesis, we would like to study (i) the potential role of this second EIIA protein in the regulation of SpoT, (ii) to characterize the regulations of both EIIA proteins. On the other hand, we will use an unbiased high throughput approach – the Tn-Seq - to highlight potential new regulators of SpoT.

Results

III. Results

A. Regulation of EIIA^{Ntr} protein levels

Our laboratory recently showed that, upon nitrogen starvation, the phosphorylation of PTS^{Ntr} and (p)ppGpp levels increased concomitantly [44]. This correlation is notably explained by the fact that, once phosphorylated, EIIA^{Ntr}~P interacts with SpoT to specifically inhibit its hydrolase activity, thereby avoiding degradation of (p)ppGpp. Since (p)ppGpp interferes with cell cycle progression, the G1/swarmer lifetime is strongly elongated when nutrients become limiting. Therefore, we wondered whether EIIA^{Ntr} and SpoT protein levels were fluctuating when bacteria are starved for nutrients and/or during the cell cycle of unstarved cells.

1. The EIIA^{Ntr} protein levels are stable upon nutrients starvation

To test whether EIIA^{Ntr} proteins levels change upon starvation, we diluted an overnight culture of the *Caulobacter crescentus* WT strain (NA1000) in synthetic media without carbon (M2), without nitrogen (P2G) or with both nitrogen and carbon sources (M2G). M2 and P2G media were previously shown to induce (p)ppGpp accumulation [44], [47], [50]]. As a control, NA1000 was also diluted in a complex rich medium (PYE). The resuspended cells were then incubated at 30° C with agitation and protein samples withdrawn after 5 minutes and 1 hr of incubation. Then, samples were separated by SDS-PAGE and levels of EIIA^{Ntr}, SpoT and the actin-like MreB (used a loading control) were evaluated by western blot using specific antibodies. Note that anti-EIIA^{Ntr} antibodies were produced during this Master thesis (Figure 9A). Both EIIA^{Ntr} and SpoT were found to be stable when cells were incubated without a nitrogen (P2G) or carbon (M2) source (Figure 9B).

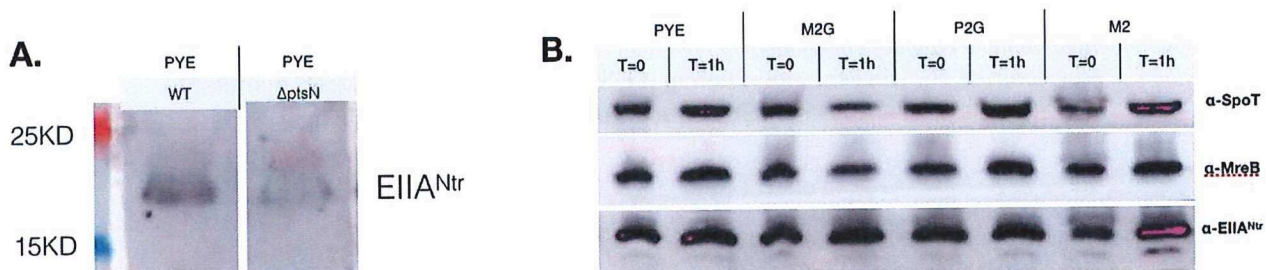


Figure 9: EIIA^{Ntr} abundance in stress conditions

- A.** Western blot testing the new polyclonal antibodies anti-EIIA^{Ntr} on a WT and $\Delta ptsN$ strains grown in PYE.
- B.** Western Blots showing levels of EIIA^{Ntr}, SpoT and MreB proteins from cultures grown under different conditions. A WT strain of *Caulobacter crescentus* was grown in complex medium (PYE), synthetic medium (M2G), synthetic medium depleted either for nitrogen (P2G) or carbon source (M2). Protein samples were taken at time 0h and 1h for each condition.

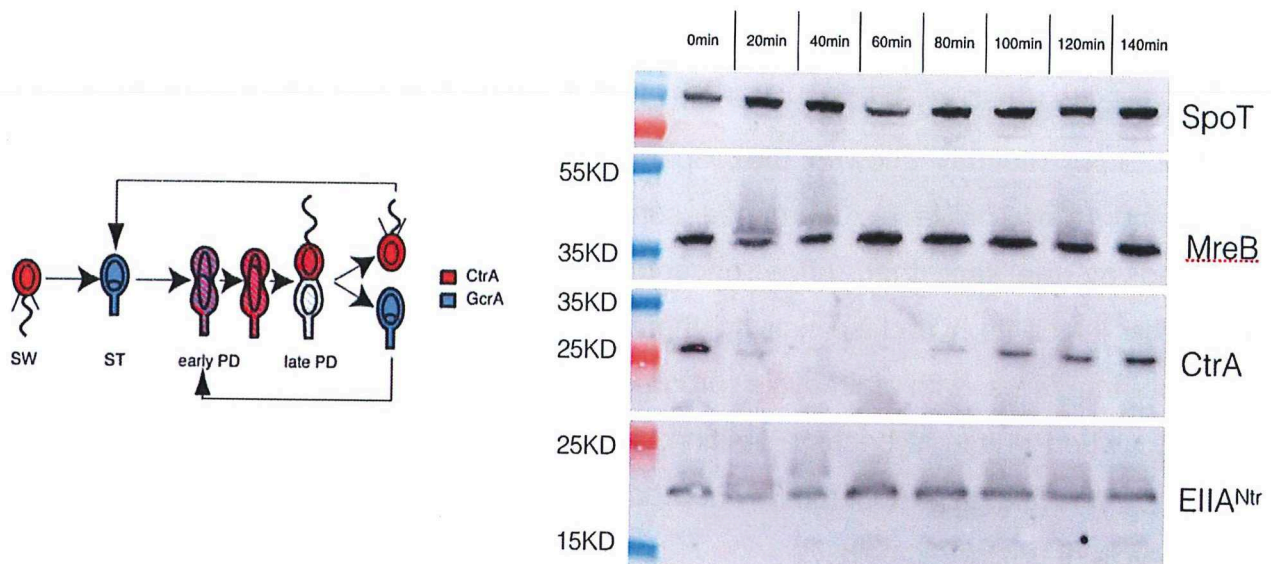


Figure 10: EIIA^{Ntr} abundance is stable throughout the cell cycle

A. Schematic representation of *C. crescentus* cell cycle. From Holtzendorff et al. 2006.

B. Western blot showing the abundance of EIIA^{Ntr}, SpoT, CtrA and MreB along the cell cycle of *C. crescentus*. A synchrony was realised on a strain grown in M2G and samples were taken every 20 minutes for 140 minutes. CtrA was used as a control of the synchrony and MreB as a loading control.

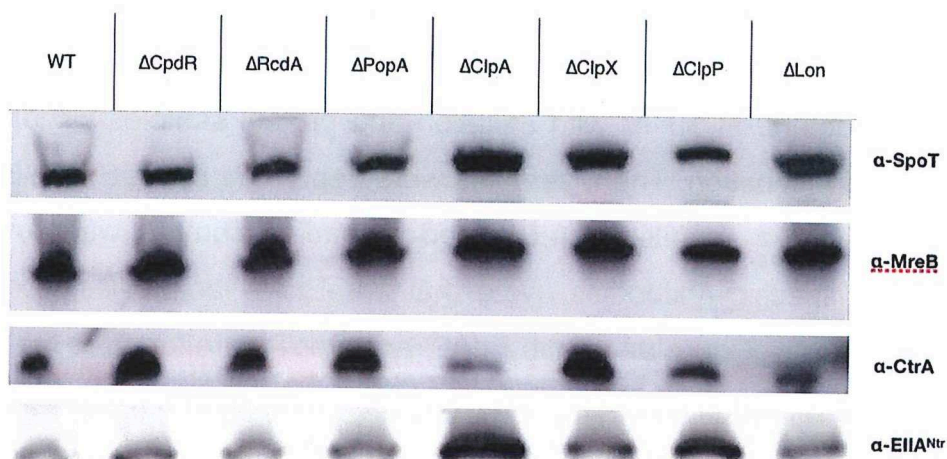


Figure 11: Proteolysis of EIIA

Western blot showing the abundance of EIIA^{Ntr}, SpoT, CtrA and MreB in mutant of proteases and adaptors of *Caulobacter crescentus*.

2. EIIA^{Ntr} does not oscillate during the cell cycle

To test whether EIIA^{Ntr} levels vary throughout the cell cycle, a WT population was synchronized by recovering and resuspending the G1 swarmer cells in a fresh M2G medium. Protein samples were taken every 20 minutes and a western blot performed to evaluate the abundance of EIIA^{Ntr} throughout the cell cycle. CtrA was used to control the quality of the synchrony since it is present in G1/swarmer cells, degraded at the G1-to-S transition before being resynthesized in late S phase [51]. As MreB is stable throughout the cell cycle, it was used as a loading control. Our data revealed that EIIA^{Ntr} as well as SpoT were stable along the cell cycle whereas CtrA oscillated as expected (Figure 10).

3. EIIA^{Ntr} is likely proteolyzed by ClpAP

Because the C-terminal extremity of EIIA^{Ntr} harbors two hydrophobic residues (Ala-Ala) [52], also found in many proteins degraded by ClpXP or ClpAP protease, we tested whether EIIA^{Ntr} is proteolyzed. For that, the levels of EIIA^{Ntr} were evaluated in proteases mutants (ClpXP, ClpAP or Lon) or in mutants of specific adaptors. Indeed, *C. crescentus* ClpXP use different adaptors (CpdR, RcdA and PopA) to selectively degrade substrates [53]. It is noteworthy that *clpX* and *clpP* genes are essential, because ClpXP degrades the SocB toxin of the SocAB toxin/antitoxin system [54]. Thus, the absence of ClpX or ClpP leads to toxic SocB accumulation. Therefore, mutant alleles of *clpX* ($\Delta clpX$) and *clpP* ($\Delta clpP$) were generated in a strain devoid of SocAB ($\Delta socAB$).

The WT and mutant strains were grown in complex medium (PYE) until mid-exponential phase of growth ($OD_{660} \sim 0.2-0.5$). Then, samples were withdrawn and proteins extracts separated by SDS-PAGE. Finally, levels of EIIA^{Ntr}, SpoT, CtrA and MreB were determined by western blot. CtrA was used as a positive control since it is degraded by ClpXP in a CpdR-, RcdA- and PopA-dependent way whereas MreB was used as a loading control. Interestingly, we found that EIIA^{Ntr} was more abundant in the *clpA* - and *clpP*- mutant strains (Figure 11; EIIA^{Ntr}/MreB ratio ≥ 200 % of the WT levels), suggesting that EIIA^{Ntr} is degraded by the ClpAP protease. Accordingly to the literature, the CtrA/MreB ratios were (i) unchanged in Δlon (~ 110 % of the WT levels), (ii) lower in $\Delta clpA$ (~ 60 % of the WT levels) and (iii) higher in all the other mutant strains (~ 130 % - 180 % of the WT levels) than in the WT [53].

B. Regulation of SpoT by EIIA

As stated in the Introduction, *C. crescentus* harbors a hybrid PTS/PTS^{Ntr}, including (i) EI^{Ntr} and EIIA^{Ntr} belonging to the PTS^{Ntr} and (ii) EIIA and HPr from the canonical PTS.

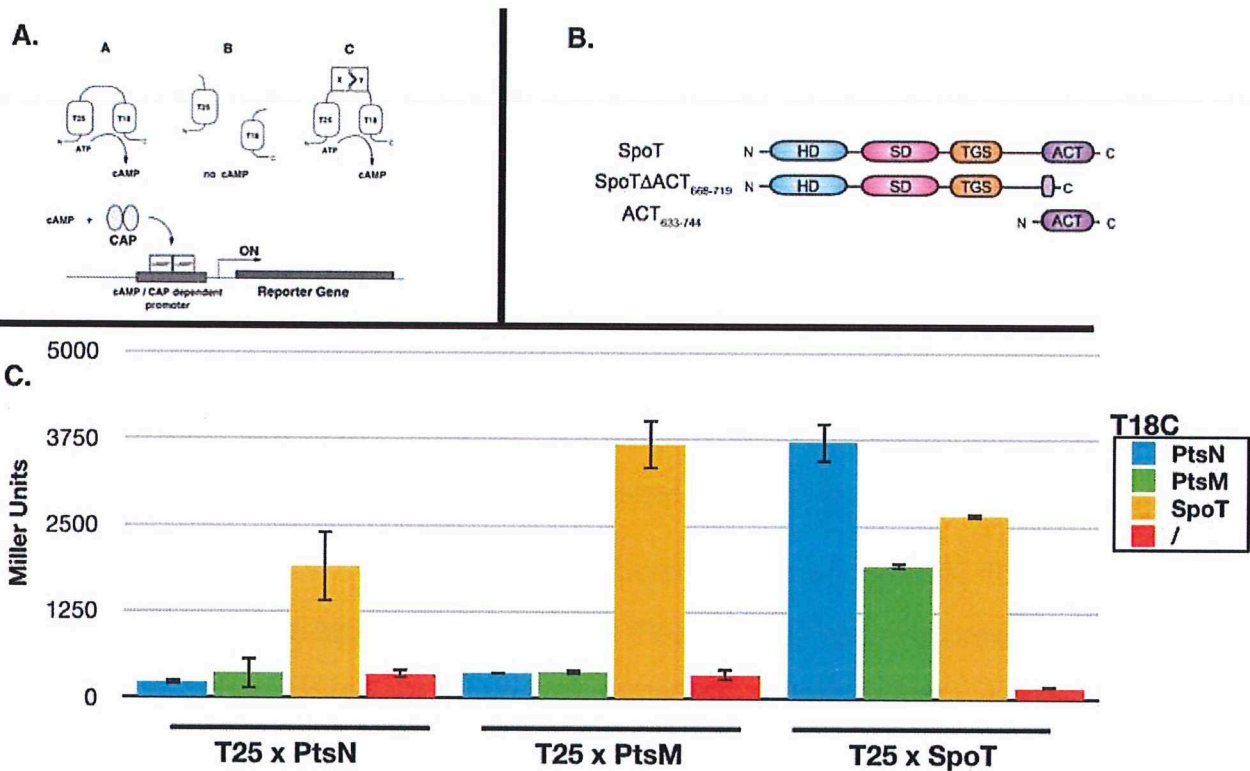


Figure 12: EIIA interacts with SpoT

A. Schematic representation of the BTH assay. The T25 and T18 domains of the cAMP are fused to proteins X and Y. When proteins interact with each other, a functional hybrid adenylate cyclase is reconstituted, allowing cAMP production and subsequent transcriptional activation of reporter genes.

Adapted from Karimova et al. 1998 [52]

B. Schematic representation of the different truncated versions of SpoT.

C. Graphical representation of BTH β -galactosidase assays showing potential interactions between SpoT and EIIA or EIIA^{Ntr}. MG1655 Δ cya::frt harbouring pKT25-spoT, pKT25-ptsN or pKT25-ptsM was transformed with pUT18C-spoT, pUT18C-ptsN or pUT the 18C-ptsM but also empty plasmid as a negative control. Error bars = s.d with n = 3.

Although EIIA^{Ntr}, HPr and EIIA^{Ntr} have been described to regulate (p)ppGpp levels by modulating SpoT activities [44], the function of EIIA has not been elucidated yet. As the gene coding for EIIA (*ptsM*) is part of the same operon than the gene coding for HPr (*ptsH*), it suggests that EIIA might also regulate SpoT activity.

1. EIIA interacts with SpoT

As EIIA^{Ntr} was demonstrated to physically interact with SpoT [44], we tested whether EIIA was also able to interact with SpoT in a bacterial two-hybrid (BTH) assay. The BTH assay is based on the reconstitution of a functional adenylate cyclase (AC) from two separate domains (T18 and T25) fused to proteins (X and Y) that can interact with each other [55]. Thus, when the hybrid proteins (T18-X and T25-Y) interacts with each other, the reconstituted hybrid AC catalyses the production of cyclic adenosine monophosphate (cAMP) from ATP, which will then activate the CAP-dependent transcription of reporter genes such as *mal* and *lac* operons, respectively involved in the catabolism of maltose and lactose (Figure 12A). The consumption of maltose will acidify the medium, which will turn the Neutral Red dye contained into the MacConkey agar into a red color. In addition, we can measure the activity of the β -galactosidase (encoded by *lacZ* into the *lac* operon) with ortho-nitrophenyl- β -galactoside (ONPG), which once cleaved by the β -galactosidase will turn into a yellow color. This last assay has the big advantage to be quantitative in contrast to the qualitative MacConkey agar assay.

First, we cloned the gene coding for EIIA (*ptsM*) in the BTH plasmids pUT18C and pKT25. Then, the *E. coli* strain MG1655 Δ *cyaA::frt* was transformed with combinations of pKT25-X and pUT18C-Y plasmids, where X and Y are EIIA (*ptsM*), SpoT (*spoT*) or EIIA^{Ntr} (*ptsN*). Unfortunately, T25-EIIA autoactivated *mal* expression and gave a strong red color on McConkey agar plate even in combination with the empty pUT18c plasmid (data not shown). However, T25-EIIA did not autoactivate *lacZ* but interacted with T18-SpoT as T25-EIIA^{Ntr} did (Figure 12B). In addition, all the three T18 fusions (T18-EIIA, T18-EIIA^{Ntr} and T18-SpoT) interacted with T25-SpoT (Figure 12B). These data suggest that both EIIA proteins can interact with SpoT.

2. The phosphorylated form of EIIA interacts with SpoT

To further characterize the interaction between EIIA and SpoT, the phosphomimetic (EIIA_{H9E}) and the non-phosphorylatable (EIIA_{H9A}) version of EIIA were tested in the BTH

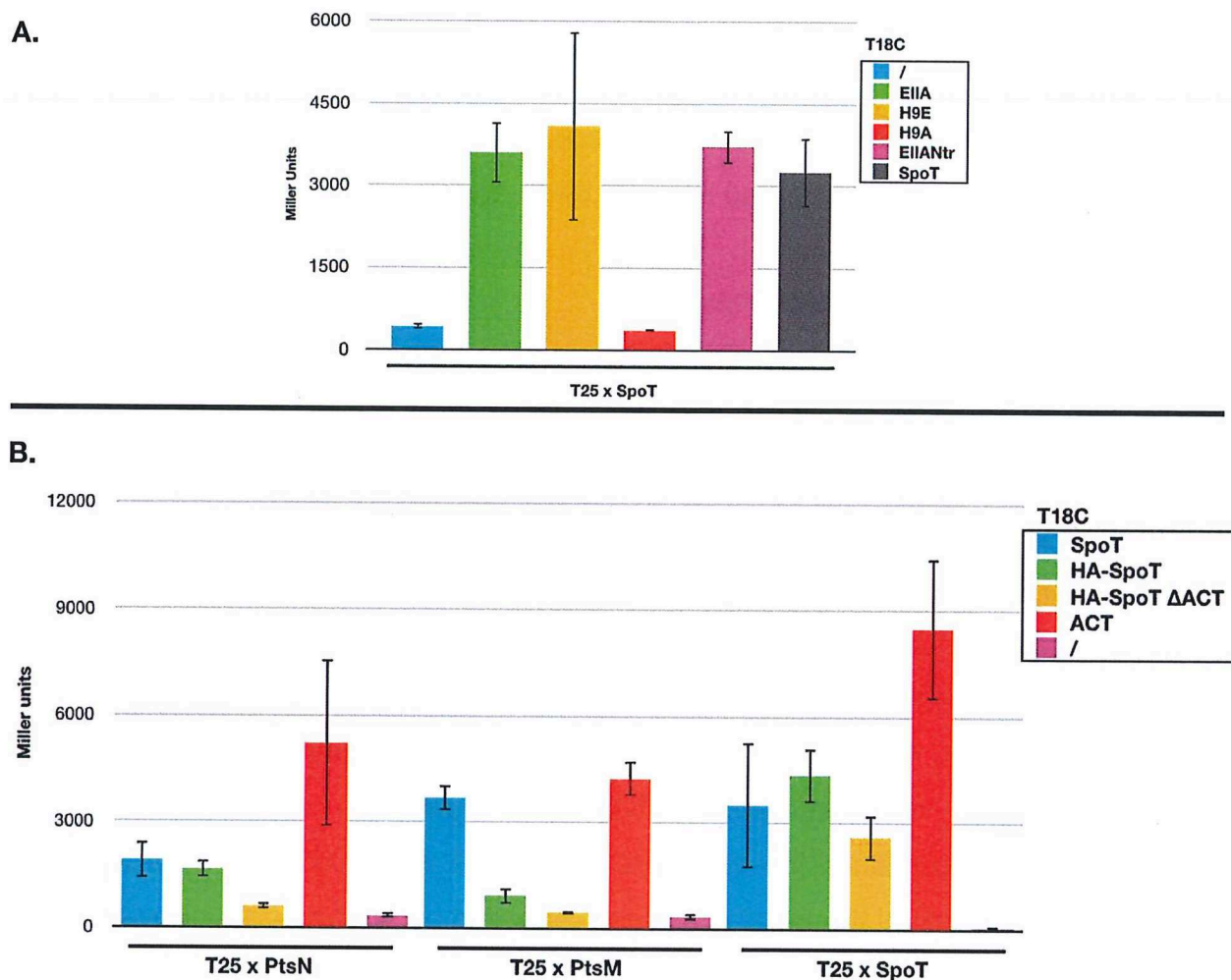


Figure 13: The phosphorylated version of EIIA likely interacts with the ACT domain of SpoT

A. β -galactosidase BTH assays measuring the potential interaction between the SpoT and EIIA, EIIAH9E, EIIAH9A, EIIANtr or SpoT. MG1655 $\Delta cya::frr$ harbouring pKT25-*spoT*, was transformed with empty pUT18C, pUT18C-*ptsM*, pUT18C-*ptsM*_{H9E}, pUT18C-*ptsM*_{H9A}, pUT18C-*ptsN*. The empty pUT18C plasmid was used as a negative control while the T18C-*spoT* served as positive control since it has been demonstrated to interact with SpoT. Error bars = s.d with n = 3.

B. β -galactosidase BTH assays measuring the potential interaction between the ACT domain of SpoT and EIIA or EIIANtr. MG1655 $\Delta cya::frr$ harbouring pKT25-*spoT*, pKT25-*ptsN* pKT25-*ptsM* were transformed with pUT18C-*spoT*, pUT18C-*ptsN*, pUT18C-*ptsM*, pUT18C-*spoT* Δ ACT or pUT18C-ACT. The empty T18C plasmid was used as a negative control and T25-SpoT as a positive control since its interaction has been demonstrated with T18C-EIIANtr, T18C-EIIA and T18C-SpoT. Error bars = s.d with n = 3.

Values expressed in Miller units are the mean of 3 independent measures.

assay. For that, *ptsM_{H9E}* and *ptsM_{H9A}* were cloned in the pUT18C and pKT25 and the interaction with SpoT by performing a β -galactosidase assay. We found that EIIA_{H9E} was still able to interact with SpoT whereas this interaction was lost with EIIA_{H9A} (Figure 13A).

3. The ACT domain of SpoT is required for the interaction with EIIA and EIIA^{Ntr}

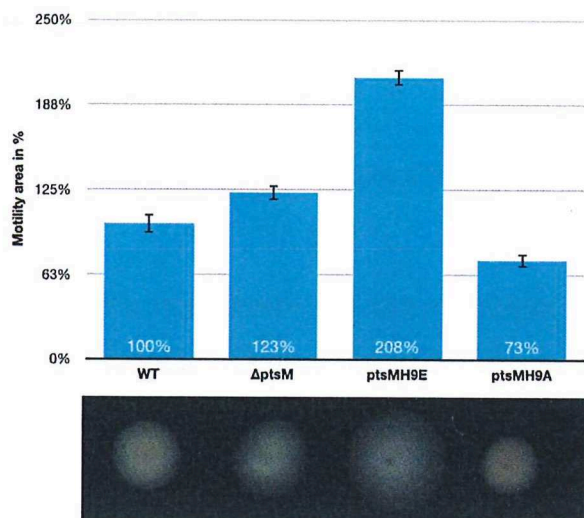
Then, we tried to identify the domain(s) of SpoT important for the interaction with EIIA. SpoT is composed of four domains: a Hydrolase Domain (HD), a Synthetase Domain (SD) and two regulatory domains TGS and ACT (Figure 13B) [27]. Interestingly, the deletion of ACT (T25-SpoT Δ ACT) completely abolished the interaction with both EIIA proteins fused to T18 (T18-EIIA^{Ntr} and T18-EIIA) but not with T18-SpoT. In contrast, the ACT domain alone (T25-ACT) strongly interacted with EIIA^{Ntr}, EIIA and SpoT fused to T18 (Figure 13B). Together, these data suggest that the ACT domain is required and sufficient to mediate the interaction with EIIA proteins.

C. EIIA regulates the G1/swarmer lifetime

Increasing (p)ppGpp concentration delays the G1-to-S and swarmer-to-stalked cell transitions [10], [44]]. As a consequence, the growth rate decreases whereas the G1 population and the motility increase. To study the potential implication of EIIA in regulating SpoT activities, we first measured the doubling time, the motility and the G1 proportion of several EIIA mutants: a KO mutant (Δ *ptsM*) but also two point mutants interfering with the phosphorylation of EIIA (*ptsM_{H9E}* and *ptsM_{H9A}*).

To evaluate the impact of *ptsM* mutations on growth, the WT, Δ *ptsM*, *ptsM_{H9E}*, *ptsM_{H9A}* strains were grown in PYE complex medium and the OD₆₆₀ was measured every 10 minutes for 24 hours (Figure 14A). Interestingly, both Δ *ptsM* and *ptsM_{H9E}* strains grew slower than the WT whereas *ptsM_{H9A}* cells grew as fast as the WT but reached a higher plateau than the WT. These phenotypes were already observed with *spoT* mutants since the HD dead mutant *spoT_{D81G}* accumulated (p)ppGpp and grew slowly whereas the KO mutant Δ *spoT* grew at a higher plateau in stationary phase [44]. Interestingly, the phosphomimetic and phospho-dead mutants of EIIA^{Ntr} (*ptsN_{H66E}* and *ptsN_{H66A}*, respectively) also displayed opposing growth behaviours, with a slower growth for EIIA^{Ntr_{H66E}} and a higher plateau for EIIA^{Ntr_{H66A}} (Figure 14B) [44]. However, a knock-out mutant of EIIA^{Ntr} (Δ *ptsM*) phenocopied the unphosphorylatable mutant (*ptsN_{H66A}*). Thus, in contrast to EIIA^{Ntr}, which inhibits growth under its phosphorylated form (by increasing

A.



B.

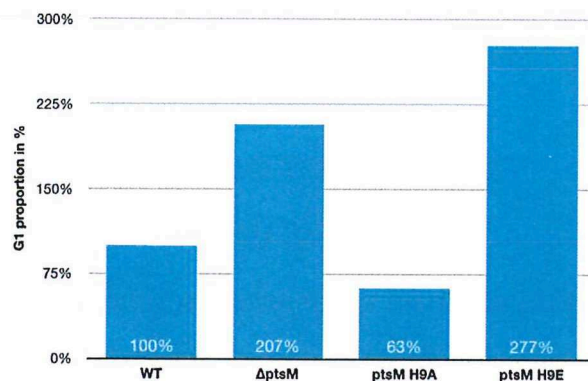
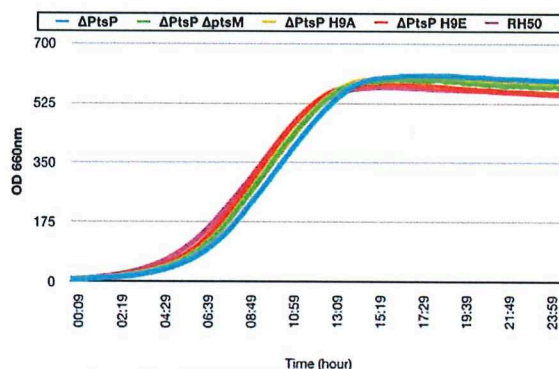


Figure 15: Phenotypes displayed by *ptsM* mutants

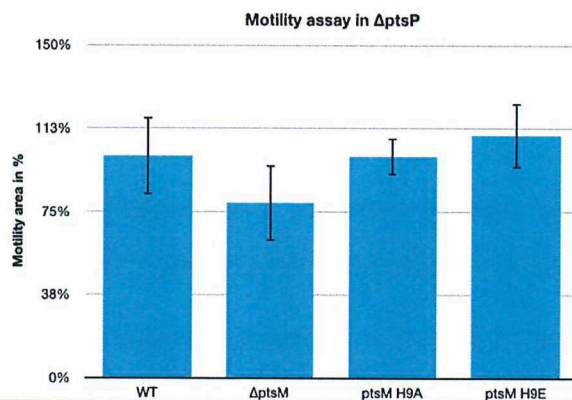
A. Motility assays showing EIIA mutants on PYE swarmer plates. Values, expressed as relative areas (in percent), are the mean of 3 independent measurements. Error bars = s.d with $n = 3$.

B. Proportion of G1 cells in EIIA mutants populations. DNA of heterogenous populations of each indicated strain grown in exponential phase was labelled with sytox-green. DNA content was then measured by FACS revealing cells containing 1 DNA copy (G1), 2 DNA copies (G2) or replicating cells (S).

A.



B.



C.

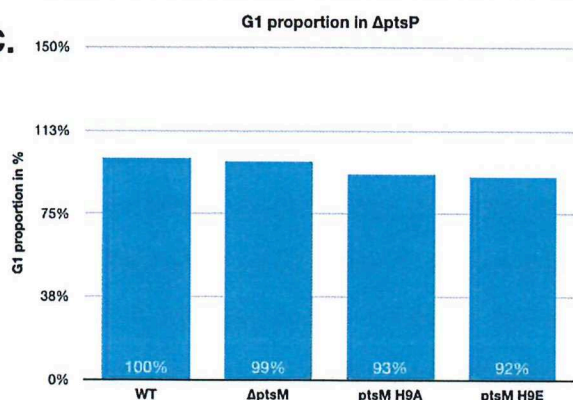


Figure 16: Suppression of the phenotypes displayed by *ptsM* mutants in a Δ ptsP background

A. Growth curves of WT and *ptsM* mutants, in a *ptsP* background. Strains were grown in PYE medium and the OD₆₆₀ were measured every 10 minutes during 24 hours.

B. Motility assays with *ptsM* mutants in a Δ ptsP background. Values expressed relative areas in percent are the mean of 3 independent measurements. Error bars = s.d with $n = 3$.

C. G1 proportion of *ptsM* mutants in a Δ ptsP background. DNA of heterogenous populations of each indicated strain grown in exponential phase was labelled with sytox-green. DNA content was then measured by FACS revealing cells containing 1 DNA copy (G1), 2 DNA copies (G2) or replicating cells (S).

(p)ppGpp levels), our data show that the unphosphorylated form of EIIA is required for optimal growth. EIIA might therefore be an inhibitor of SpoT.

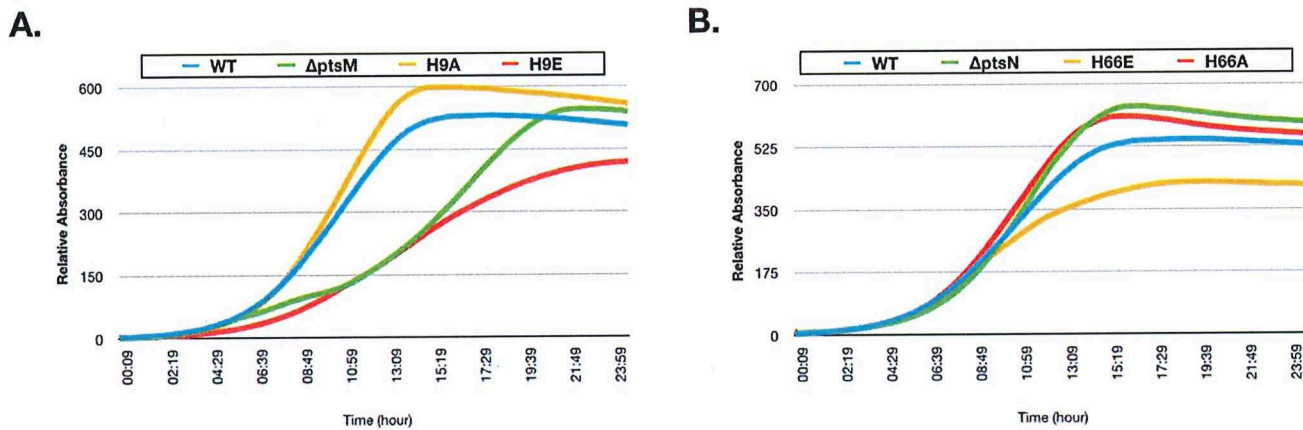


Figure 14: Growth curves of WT and mutants of *ptsM* and *ptsN*.

The different strains were cultured in PYE medium and the OD₆₆₀ were measured every 10 minutes during 24 hours.

A. $\Delta ptsM$ and $ptsM_{H9E}$ mutants show a lower growth rate in comparison to the WT, whereas $ptsM_{H9A}$ had a higher plateau.

B. $ptsN_{H66E}$ and $ptsN_{H9A}$ grew respectively slower and faster in comparison to the WT.

As a potential regulator of SpoT, EIIA should also interfere with the G1-to-S transition and thereby increase motility and G1 proportion. To test that, we measured the motility on swarmer agar plates and the G1 proportion by flow cytometry of WT, $\Delta ptsM$, $ptsM_{H9E}$ and $ptsM_{H9A}$ cells (Figure 15A and 15B). Accordingly to our expectation we found that $\Delta ptsM$ and $ptsM_{H9E}$ displayed a bigger motility halo and G1 fraction whereas $ptsM_{H9A}$ was less motile and spent less time in G1 phase than the WT (Figure 15A and 15B).

Together, these data support the idea that EIIA is required for growth, perhaps by inhibiting SpoT-dependent production of (p)ppGpp.

To further test this hypothesis, we studied the effects of the *ptsM* mutations ($\Delta ptsM$, $ptsM_{H9E}$ and $ptsM_{H9A}$) in a $\Delta ptsP$ background. Surprisingly, the deletion of *ptsP* completely suppressed the defects generated by $\Delta ptsM$ and $ptsM_{H9E}$, since all $\Delta ptsP$ strains had similar growth, motility and G1 proportion whatever the *ptsM* allele was expressed (Figure 16A-C). This result seems incompatible with our hypothesis since deletion of *ptsP* cannot interfere with the phosphorylation state of EIIA_{H9E}. For instance, the phenotypes displayed by EIIA^{Ntr}_{H66E} are not fully suppressed by $\Delta ptsP$ [44].

D. Tn seq experiments to identify potential new SpoT regulators

To further characterize the regulation of SpoT in *C. crescentus*, we performed a Tn-Seq experiment in the WT, $spoT_{\Delta ACT}$ and $\Delta spoT$ strains. These 3 strains have different

intracellular (p)ppGpp levels in unstressed conditions. Whereas $\Delta spoT$ cells do not produce (p)ppGpp, the $spoT_{\Delta ACT}$ strain accumulates (p)ppGpp because the hydrolase activity requires the ACT domain (Ronneau et al., submitted). Therefore, the $spoT_{\Delta ACT}$ strain has a growth delay when grown in complex PYE medium. Interestingly, inactivating *ptsP* (EIN^{tr}) or introducing the Y323A¹ substitution into $spoT_{\Delta ACT}$, suppressed the growth delay (Ronneau et al., submitted), showing that the high (p)ppGpp levels in $spoT_{\Delta ACT}$ cells was responsible for the growth delay. Indeed, HPr~P has been shown to be required for sustaining the synthetase (SD) activity of SpoT and HPr is no longer phosphorylated in $\Delta ptsP$ cells. We reasoned that any transposon (Tn) insertions disrupting gene function indispensable for SpoT SD activity should suppress the growth defects of $spoT_{\Delta ACT}$ without perturbing growth of the WT and the $\Delta spoT$ strains, and thus should be favoured in the $spoT_{\Delta ACT}$ background. Likewise, inactivating of inhibitor of the SpoT SD activity should specifically aggravate the growth defects of $spoT_{\Delta ACT}$. A Tn-seq is a genome-wide mutagenesis, which consists to saturate the genome with transposon (Tn) and to sequence Tn insertion sites by Illumina sequencing. Based on our prediction, Tn insertions

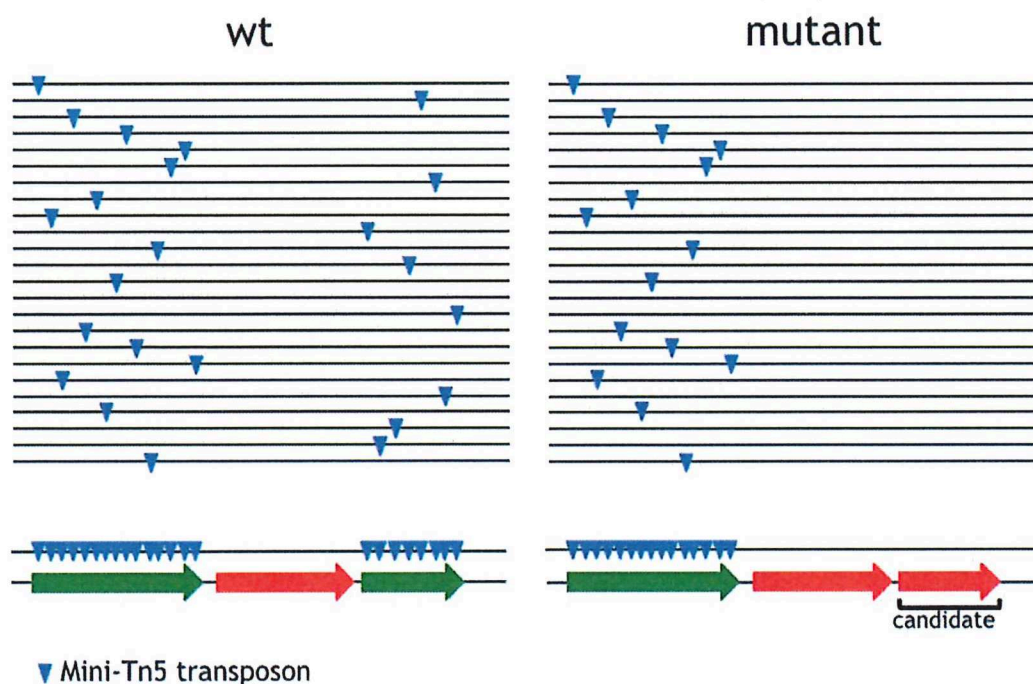


Figure 17: Schematic representation of Tn-seq experiments. Insertions of transposon are represented by blue arrow while genome of bacteria is represented by the black lines. The lower image represents the mapping of the transposons on the genome highlighting potential candidates while comparing the WT (left) and the mutant (right).

alleviating the growth defects of $spoT_{\Delta ACT}$ should accumulate in this background in

¹ The Y323A substitution inactivates the synthetase activity of SpoT [50]

comparison to the WT and $\Delta spoT$ backgrounds, whereas Tn insertions into genes coding for SpoT inhibitors should be under-represented in $spoT_{\Delta ACT}$ (Figure 17).

To perform Tn-seq, we generated a library of more than 300,000 transpositional mutants in each background on PYE agar plates. Then, genomic DNA was recovered from each library and sequenced by Illumina sequencing (single reads), and the reads mapped on the *C. crescentus* reference genome (NA1000, NC_011916.1). The number of reads per gene was then calculated. Since it has been shown that the extremities (5' and 3') of a gene are often dispensable, we did not take into account the reads corresponding to the first and last 10% of each gene in the calculation. Finally, these values were used to calculate the ratio (of Tn insertions per gene) between each mutant strain ($spoT_{\Delta ACT}$ or $\Delta spoT$) and the WT, so that any increase or decrease of Tn insertions into a specific gene would give a ratio respectively higher or lower than 1.0. The thresholds of 1.5 and 0.5 allowed us to identify potential regulators (activators or inhibitors) of SpoT SD activity (Table 1). As stated above, the inactivation of *ptsP* (EIN^{tr}) or *spoT* itself suppressed the growth defects of $spoT_{\Delta ACT}$. As expected, Tn insertions into *ptsP* gene accumulated in $spoT_{\Delta ACT}$ cells (ratio of 6.1), but also, to a lesser extent, in $\Delta spoT$ cells (ratio 3.2). Tn insertions into *spoT* gene also accumulated in $spoT_{\Delta ACT}$ cells, but to a lesser extent (ratio of 1.6).

Discussion & perspectives

IV. Discussion

A. EIIANtr abundance during cell cycle proteolysis and stress condition?

1. EIIANtr is stable throughout the cell cycle

The abundance of the (p)ppGpp has been shown to vary in stress conditions in bacteria. For instance, it accumulates when *Escherichia coli* cells are starved for carbon, nitrogen, phosphate, iron, fatty acids or amino acids but also under a heat shock [3], [56]–[59]]. In *Caulobacter crescentus*, (p)ppGpp accumulates only upon nitrogen or carbon starvation, which results in a drastic reorganisation of the cell, notably by delaying cell cycle progression, improving motility and slowing the growth [44], [46], [60]–[62]]. These effects are likely due to a transcriptional and/or translational reprogramming. This stress response has been proposed to be critical in natural environment since upon nutrient starvation, a high concentration of (p)ppGpp will maintain the swarmer cells in G1 phase until environmental conditions are again favourable, thereby avoiding irreversible differentiation into sessile stalked cells and entry into the cell cycle. Indeed, the motile swarmer cells can still move away from the poor environment.

Beside this role as a stress response molecule, (p)ppGpp is also a cell cycle regulator since (p)ppGpp⁰ cells ($\Delta spoT$) will spend less time in G1/swarmer phase even in a complex rich media. In light of this result, it has been proposed that (p)ppGpp levels might oscillate along the cell cycle. Interestingly, another hyperphosphorylated guanosine – the second messenger cyclic-di-GMP (cdGMP) – has been shown to oscillate during the cell cycle with a peak in concentration at the G1-S transition [61], [63]. In *C. crescentus*, cdGMP stimulates the G1-S and swarmer-to-stalk transitions pushing the swarmer cell to abandon motility and to start DNA replication. More specifically, cdiGMP stimulates ejection of the flagellum, elongation of the stalk and synthesis of the holdfast [64]–[67]]. It also interacts with the cell cycle kinase CckA, triggering inactivation of the DNA replication inhibitor CtrA. Similarly to cdGMP, (p)ppGpp might also fluctuate throughout the cell cycle to peak in concentration during G1. In addition, it was shown that (p)ppGpp inhibits the activity of the YybT protein, a phosphodiesterase that hydrolyses cyclic di-AMP and cdiGMP [68]. This suggests there are cross-links between (p)ppGpp and cdGMP pathways that might also exist in *C. crescentus*. The similarities and the possible crosstalk between the two second messengers (cdGMP and (p)ppGpp) led us to investigate the possible oscillation of (p)ppGpp along the cell cycle in *C. crescentus*.

We first tried to show the (p)ppGpp abundance over time in *C. crescentus* using a high-performance liquid chromatography (HPLC) quantification as it was previously done in *E. coli* [69]. The protocol was divided in 3 steps: (i) for the acquisition, we harvest the cells by pouring culture in formic acid and then rapidly quench the bacteria (see Material and Methods). The filtration was used to avoid centrifugation known to interfere with stability of nucleic acids; (ii) nucleic acids were then extracted chemically in cold formic acid and separated on a fast protein liquid chromatography (FPLC) column and (iii) finally, nucleic acids were quantified by HPLC. We started with some controls by comparing a WT *C. crescentus* strain grown in replete or deplete (-N) conditions to the $\Delta spoT$ strain cultivated in the same conditions. Unfortunately, our attempts to quantify (p)ppGpp remained unsuccessful due to a (too) low detection level of the molecule. It would be interesting to use the same approach that the one used to quantify cdGMP, that is a HPLC coupled to mass spectrometry [63], [70]. Another possibility would be to use the specific probe binding (p)ppGpp one of our collaborators designed. This fluorescent probe could be used to evaluate (p)ppGpp levels at the single cell level during the cell cycle of *C. crescentus*.

Then, we followed the cell cycle abundance of the unique (p)ppGpp synthetase/hydrolase protein (SpoT) as well as its physical partner (EIIA^{Ntr}). EIIA^{Ntr} is known to repress the hydrolase activity of SpoT and, in consequence, to regulate the levels of (p)ppGpp not only in stress conditions but also in unstressed conditions [44]. An oscillation of at least one of this two proteins could further suggest a spatiotemporal regulation of (p)ppGpp during the cell cycle. However, we did not detect any fluctuation in the abundance of SpoT or EIIA^{Ntr}. Although levels of EIIA^{Ntr} do not oscillate along the cell cycle, its phosphorylation could be regulated throughout the cell cycle and drive oscillation of (p)ppGpp levels. Indeed, it has been shown that only the phosphorylated form of EIIA^{Ntr} (EIIA^{Ntr}~P) could interact with SpoT to inhibit its HD activity. Therefore, it would be interesting to measure levels of EIIA^{Ntr}~P along the cell cycle by immunoprecipitating EIIA^{Ntr} from a radio-labelled synchronized population of WT cells. Note that the levels of EI^{Ntr} was shown to be stable throughout the cell cycle and that we were not able to determine the levels of HPr since it was not detectable neither with polyclonal antibodies nor with anti-tag antibodies. This latter data suggests that HPr levels are very limiting.

2. EIIA^{Ntr} is stable in stress conditions

As mentioned above, (p)ppGpp accumulate under stress conditions in *C. crescentus* [3], [44]]. This accumulation leads to a delay in the G1-S phase transition keeping the bacteria at the swarmer stage [44], [71]. EIIA^{Ntr} abundance could increase while the bacteria were

grown in stringent conditions (M2G, M2, P2G). To test this hypothesis, a western blot was performed on *C. crescentus* incubated for 1hr in media depleted for carbon or nitrogen source. Note that (p)ppGpp levels strongly increase in these conditions. The abundance of EIIA^{Ntr} was shown to be stable in every stress conditions, suggesting that EIIA^{Ntr} levels do not increase upon stress. Since the phosphorylation state of EIIA^{Ntr} has been shown to increase under stress conditions [44], our results suggest that the protein levels of EIIA^{Ntr} is likely sufficient to sustain the stress response.

3. ClpAP-mediated degradation of EIIA^{Ntr}

The C-terminal end of EIIA^{Ntr} suggested that this protein could be proteolysed. To find the protease potentially degrading EIIA^{Ntr}, we performed western blots on mutants of the proteases subunits (ClpX, ClpP, ClpA) and adapters (PopA, RcdA and CpdR). Our results revealed EIIA^{Ntr} abundance increased in a ClpAP-dependent way. Interestingly, ClpAP was shown to degrade the essential protein DnaA (also degraded by Lon) and FliF [72]–[74]]. DnaA is a highly conserved initiator of the DNA replication, which promotes unwinding of the *oriC* as well as the assembly of the replisome at the *oriC* [75], [76]. To trigger DNA replication at the right time, DnaA is highly regulated especially by degradation. In consequence DnaA oscillates to reach the maximal level after the differentiation of the swarmer cells into stalked cells [77]. Furthermore, the high levels of (p)ppGpp produced during carbon limitation promote the degradation of DnaA to inhibit the DNA replication [47]. Finally, the ClpAP-dependent degradation of DnaA has been shown to proceed in stationary phase, that is when (p)ppGpp levels increase. Thus, all the data converge to a model indicating that the ClpAP-dependent degradation of DnaA relies on (p)ppGpp and it is intriguing to see that ClpAP is also responsible for the degradation of EIIA^{Ntr}, a protein inhibiting (p)ppGpp degradation. Nevertheless, we did not find yet any conditions that trigger degradation of EIIA^{Ntr}. The MS ring protein FliF anchors the flagellar structure in the inner membrane and is located at the flagellated pole of *C. crescentus*. The proteolysis of FliF is also regulated during the cell cycle, the degradation being triggered in differentiating cells to facilitate the ejection of the flagellum [72], [78]. Moreover, the degradation of FliF requires the presence of PleD, a cdGMP synthetase [79], [80]. Whereas DnaA proteolysis by ClpAP is induced by (p)ppGpp, it seems that FliF degradation is inhibited by the alarmone but stimulated by cdGMP. It is tempting to speculate that second messengers drive ClpAP-dependent proteolysis along the cell cycle. Such hierarchy could involve adapters specific for each signalling molecules. Interestingly, it has been shown that

proteolysis of CtrA relies on ClpXP and requires not only the 3 adapters (PopA, RcdA and CpdR) but also of cdGMP bound to PopA.

ClpS is the only adapter for ClpAP identified so far, capable to induce or inhibit ClpAP-dependent degradation of proteins [81], [82]. It would be interesting to test whether (p)ppGpp regulate directly ClpA or if ClpS is required, by performing a western blot on EIIA^{Ntr} in a $\Delta clpS$ strain.

B. Regulation of SpoT by EIIA

1. EIIA is interacting with SpoT

In 2016, Ronneau et al. identified the PTS in *C. crescentus* as a regulatory mechanism involved in nitrogen stress. The study described the function of EI^{Ntr}, HPr, and EIIA^{Ntr} regulating the activity of SpoT [44]. However, the protein EIIA may also be part of the regulatory PTS but its function and regulation remained unknown. Our results revealed an interaction between EIIA and SpoT through the ACT domain, similarly to EIIA^{Ntr}. As described in the introduction, SpoT contains both (p)ppGpp synthetase and hydrolase domains as well as two regulatory domains (TGS, ACT) [27]. EIIA^{Ntr} interacts with SpoT when phosphorylated leading to the inhibition of the SpoT hydrolase activity [44]. We hypothesised that intramolecular interactions of SpoT induce a conformational change that inhibits its SD activity and activates its HD activity. In contrast, the binding of regulatory proteins activates its SD activity and inhibits its HD activity.

The interaction of EIIA with SpoT could act as a repressor or an activator of SpoT. The fact that a phosphomimetic mutant of EIIA (EIIA_{H9E}) still interacted with SpoT whereas the phospho-dead mutant (EIIA_{H9A}) did not is reminiscent to what was described for EIIA^{Ntr}, with EIIA^{Ntr}_{H66E} interacting with SpoT and EIIA^{Ntr}_{H66A} unable to interact with SpoT. These results suggest that the phosphorylation of both EIIA proteins enhances interaction with SpoT. However, we have to be careful with our conclusions since we cannot exclude the possibility that the expression of *Caulobacter* EIIA (*Cc*EIIA) does not interfere with the endogenous PTS system of *E. coli*. Indeed, the BTH assay is based on the functional reconstitution of an adenylate cyclase allowing production of cAMP. Once produced, cAMP then triggers the transcription of catabolic operons such as those involved in lactose or maltose catabolism. But the uptake of these sugars has been shown to depend on the PTS [11]. Thus, it is possible that the expression of *Cc*EIIA interferes with uptake of maltose, especially the EIIA_{H9A} mutant since the unphosphorylated form of *E. coli* EIIA is known to inhibit sugar uptake. Nevertheless, the β -galactosidase assay does not require

uptake of carbohydrates, we are thus confident about the interaction observed between EIIA and SpoT. Nonetheless, this interaction should be validated with another technique, like a co-immunoprecipitation.

2. EIIA regulates the G1/Swarmer transition

As our data showed an interaction between EIIA and SpoT, we suspect that the protein regulates SpoT activity and thus (p)ppGpp levels. In consequence we studied the implication of EIIA mutants on phenotypes shown to be directly dependent of the levels of (p)ppGpp. To this end, we evaluated the growth, the motility and the G1 proportion of $\Delta ptsM$ (EIIA⁻) and $\Delta ptsN$ (EIIA^{Ntr}-) cells and found opposite effects. Indeed, the $\Delta ptsM$ strain grew slower than the WT, accumulated in G1 and increased its motility whereas the $\Delta ptsN$ strain had a lower G1 proportion and motility behaviour in comparison to the WT. From these first results, we can hypothesize that EIIA and EIIA^{Ntr} regulate SpoT activity in an opposite way. Surprisingly, the phospho-mutants of EIIA and EIIA^{Ntr} have similar phenotypes in the conditions tested (Figures 14-16). Thus, the expression of $ptsM_{H9E}$ (EIIA_{H9E}) phenocopied $\Delta ptsM$ and $ptsN_{H66E}$ (EIIA^{Ntr}_{H66E}) while the EIIA_{H9A} and EIIA^{Ntr}_{H66A} strains behave similarly with each other. These data suggest that the phosphorylated form of EIIA might activate the SD activity and/or repress the HD activity of SpoT while the unphosphorylated form could inhibit (p)ppGpp synthesis and/or activate its degradation. Taking into account that (i) only the phosphorylated form of EIIA could interact with SpoT and that $\Delta ptsM$ and $ptsM_{H9E}$ behave in an opposite way, none of the scenarios described above can fit with our results.

Surprisingly, the deletion of $ptsP$ (EI^{Ntr}) completely annihilated phenotypes (Growth, motility and G1 accumulation) induced by $ptsM$ (EIIA) mutations. As the first actor of the PTS, the presence EI^{Ntr} is indispensable for phosphorylating downstream PTS components, and consequently, $\Delta ptsP$ phenocopied $\Delta spoT$. If EIIA interferes directly with SpoT activities, we were expecting that phenotypes displayed by EIIA_{H9E} would be maintained even in the absence of EI^{Ntr}. Indeed, as a direct regulator of SpoT activity, EIIA_{H9E} should be insensitive to the inactivation of $ptsP$, at least partially. That is what we observed for EIIA^{Ntr} since EIIA^{Ntr}_{H66E} still accumulated G1 cells in a $\Delta ptsP$ background [44].

As an alternative model that fits with our *in vivo* results, we suggest that EIIA could rather regulate HPr instead of SpoT (Figure 18). HPr is the second actor of the PTS, receiving a phosphoryl group from EI^{Ntr} on its histidine 18 and transferring it to EIIA^{Ntr} (on histidine 66) and very likely on EIIA (on histidine 9). In addition, the ATP-dependent phosphorylation of

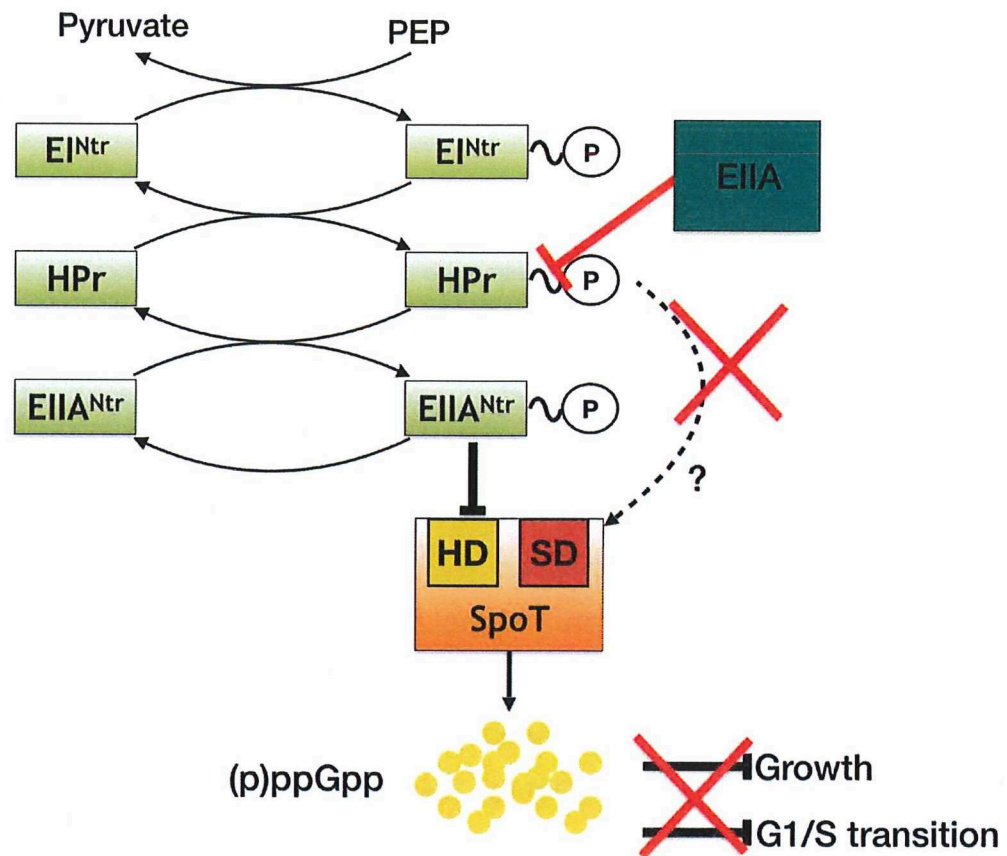


Figure 18: Alternative model of the PTS implemented by our results.

the serine 49 residue of HPr by HprK inhibits the E^{Ntr}-dependent phosphorelation on His₁₈. Considering EIIA as a repressor of HPr phosphorylation could explain the G1, motility and growth phenotypes observed in a $\Delta ptsM$ mutant. Indeed, the deletion of *ptsM* would lead to hyperphosphorylation of HPr^{His~P}, which is known to activate SpoT SD activity in an E^{Ntr}-dependent way. On the other hand, the phosphomimetic mutant EIIA_{H9E} could be unable to interact with, and thus to inhibit HPr. This is at least what it was observed for EIIA^{Ntr}, since only the phospho-dead mutant EIIA^{Ntr}_{H66A} was able to interact with HPr [44]. It would be therefore interesting to test this assumption by testing the interaction between EIIA (WT and mutants) and HPr. To further validate this model, we should purify the proteins and test whether EIIA might influence the phosphorylation of HPr without influencing directly the activities of SpoT.

C. Identification of potential regulators of SpoT

The *spoT* Δ *ACT* strain cannot anymore degrade efficiently (p)ppGpp and thereby grows slowly. A Tn-Seq was performed into that strain to identify potential positive and negative regulators of SpoT activity. The disruption of a gene coding for an activator of SpoT will reduce (p)ppGpp levels in *spoT* Δ *ACT* cells and will therefore grow faster. Tn insertions into those genes will thereby be over-represented in comparison with the Δ *spoT* strain. On the contrary, Tn insertions into a gene coding for an inhibitor of SpoT will be under-represented in the *spoT* Δ *ACT* strain.

As expected, Tn insertions into *ptsP* accumulated in *spoT* Δ *ACT* cells (ratio of 6.1). Indeed, the SD activity of SpoT Δ *ACT* is still under the positive control of HPr^{His~P} and can therefore be completely turned off in a Δ *ptsP* background (S. Ronneau et al. unpublished). However, it is more surprising to observe an accumulation of Tn insertions into *ptsP* in a Δ *spoT* background, even to a lesser extent (ratio of 3.2). This is likely due to the fact that the fitness cost of inactivating *ptsP* (E^{Ntr}) is lower in a Δ *spoT* background than that in a WT background. Indeed, (p)ppGpp are undetectable in Δ *ptsP* cells [44] and we know that the alarmone is required for optimal survival. Thus, we can hypothesise that inactivating *ptsP* in a (p)ppGpp⁰ (Δ *spoT*) strain is less disadvantageous. Interestingly, *spoT* Δ *ACT* Δ *ptsP* cells still produced (p)ppGpp levels similar to the ones observed in unstarved WT cells (S. Ronneau et al. unpublished), which supports our hypothesis. Nevertheless, we have to keep in mind that the ratios we calculate from Tn-Seq experiments are based on the net results of the difference between benefits and costs.

As *spoT Δ ACT* cells accumulate high levels of (p)ppGpp, we can expect that genes coding for activators of SpoT will present a higher ratio of Tn insertions than in *Δ spoT* cells (Table 1). Nevertheless, a high ratio of Tn insertions does not necessarily mean the gene codes for a regulator of SpoT. For example, we identified two ribonucleases (III and HI) with a ratio of 18.4 and 6.7, respectively. The ribonucleases are regulators of the cytoplasmic mRNA abundance by degrading them [83], [84]. Transpositional disruption of the ribonucleases will allow housekeeping mRNA to be abundant enough in the cells to overcome the (p)ppGpp mediated silencing of housekeeping genes [85].

One of the candidates that could regulate SpoT activity is the LSU ribosomal protein L31P with a ratio of more than 400 in *spoT Δ ACT* and a ratio of only 3.6 in *Δ spoT*. Indeed, *Caulobacter* SpoT protein has been described to be associated with the 50S subunits of the ribosomes [46]3. It is tempting to speculate that L31P is necessary for the interaction with the ribosomes as well as for sustaining the SD activity of SpoT_{Cc}. The potential role of L31P in regulating SpoT activity might be conserved in *E. coli* since a positive interaction between genes coding for L31P and RelA in *E. coli* was highlighted [86]. In *E. coli*, (p)ppGpp is synthesized by 2 proteins, RelA and SpoT whereas only SpoT can hydrolyze (p)ppGpp. If L31P inhibits the SD or activates the HD activity of SpoT_{Ec}, its inactivation could increase the (p)ppGpp levels of *Δ relA* cells and thereby improve their fitness.

In contrast to activators, Tn insertions into inhibitors of SpoT in a *spoT Δ ACT* background would be rather counter-selected.

As our list could also contain candidates directly or indirectly targeted by (p)ppGpp and responsible for the growth delay, the first thing that could be done to discriminate between potential regulators of SpoT and downstream (p)ppGpp targets would be to inactivate each gene candidate in a *spoT Δ ACT* background and roughly evaluate (p)ppGpp levels by TLC. If the corresponding candidate codes for regulator of SpoT activity, (p)ppGpp levels might change. We could first focus on the 25 non-essential genes belonging to the list (Table 1). A pull-down experiment on SpoT coupled to identification by mass spectrometry could also be done to check whether one or several of the candidates can interact with SpoT.

| CCNA | Name | Essential genome | Ratio Δ ACT | Ratio Δ SpOT |
|------------|--|--------------------|--------------------|---------------------|
| CCNA_03384 | LSU ribosomal protein L31P | essential | 419,49 | 3,64 |
| CCNA_01103 | ADP-heptose--LPS heptosyltransferase | essential | 48,72 | 0,14 |
| CCNA_00758 | protein translation Elongation factor P EF-P | essential | 45,85 | 0,20 |
| CCNA_00050 | apolipoprotein N-acyltransferase | essential | 39,23 | 0,63 |
| CCNA_03428 | cytosolic protein | essential | 25,87 | 1,09 |
| CCNA_03747 | glycosyltransferase | Nonessential | 24,59 | 0,73 |
| CCNA_01791 | CTP synthase | High_Fitness_Costs | 22,94 | 1,30 |
| CCNA_00948 | hypothetical protein =SciP | essential | 19,85 | 1,23 |
| CCNA_01497 | ADP-L-glycero-D-manno-heptose-6-epimerase | essential | 19,14 | 0,40 |
| CCNA_00670 | O-antigen export system ATP-binding protein | Nonessential | 18,73 | 0,68 |
| CCNA_01630 | ribonuclease III | High_Fitness_Costs | 18,40 | 0,46 |
| CCNA_03052 | acyltransferase | essential | 15,99 | 0,38 |
| CCNA_01971 | peptidyl-prolyl cis-trans isomerase | essential | 15,80 | 1,02 |
| CCNA_01438 | heme O monooxygenase | essential | 14,84 | 0,33 |
| CCNA_02076 | protein-L-isoaspartate O-methyltransferase | essential | 14,72 | 0,50 |
| CCNA_03755 | D-glycero-D-manno-heptose-7-phosphate 1-kinase/D-glycero-D-manno-heptose-1-phosphate adenyltransferase | essential | 13,46 | 0,54 |
| CCNA_03277 | glycosyltransferase | essential | 12,50 | 0,30 |
| CCNA_01724 | hypothetical protein | essential | 11,57 | 1,44 |
| CCNA_01989 | (3R)-hydroxymyristoyl-(acyl-carrier-protein) dehydratase | essential | 11,31 | 0,04 |
| CCNA_02994 | carbamoyl-phosphate synthase large chain | Nonessential | 10,50 | 0,67 |
| CCNA_01952 | N-acetylmuramoyl-L-alanine amidase | essential | 9,61 | 0,26 |
| CCNA_02296 | diaminopimelate decarboxylase | High_Fitness_Costs | 9,32 | 0,84 |
| CCNA_01211 | hypothetical protein | essential | 9,25 | 0,87 |
| CCNA_00501 | glucosyltransferase | essential | 8,93 | 0,06 |
| CCNA_02127 | oxacillin resistance-associated protein fmtC | High_Fitness_Costs | 8,48 | 0,11 |
| CCNA_00459 | Na ⁺ /H ⁺ antiporter nhaA | High_Fitness_Costs | 8,44 | 2,90 |
| CCNA_03256 | chorismate synthase | essential | 8,32 | 1,48 |
| CCNA_00350 | hypothetical protein | High_Fitness_Costs | 7,78 | 0,89 |
| CCNA_01116 | histidine protein kinase DivJ | essential | 7,17 | 0,68 |
| CCNA_01751 | hypothetical protein | High_Fitness_Costs | 7,15 | 0,59 |
| CCNA_03476 | ribonuclease HI | Nonessential | 6,98 | 0,36 |
| CCNA_00892 | phosphoenolpyruvate-protein phosphotransferase | High_Fitness_Costs | 6,11 | 3,21 |
| CCNA_01224 | O-antigen and teichoic acid membrane transport protein | High_Fitness_Costs | 5,37 | 0,30 |

| | | | | |
|------------|--|--------------------|------|------|
| CCNA_02286 | hypothetical protein | Nonessential | 4,94 | 3,31 |
| CCNA_03080 | antitoxin protein parD-4 | High_Fitness_Costs | 4,73 | 1,60 |
| CCNA_01419 | serine hydroxymethyltransferase | Nonessential | 4,64 | 0,17 |
| CCNA_01726 | GTP-binding protein | essential | 4,60 | 3,70 |
| CCNA_03681 | ABC transporter ATP-binding protein | High_Fitness_Costs | 4,47 | 0,38 |
| CCNA_02390 | phosphoglycolate phosphatase | High_Fitness_Costs | 4,35 | 0,98 |
| CCNA_02114 | hypothetical protein | Nonessential | 4,28 | 4,16 |
| CCNA_01754 | transglycosylase associated protein | Nonessential | 4,21 | 0,70 |
| CCNA_03650 | mannose-1-phosphate guanylttransferase | essential | 4,20 | 0,51 |
| CCNA_01959 | 3-dehydroquinate dehydratase | essential | 4,08 | 0,86 |
| CCNA_03680 | aminopeptidase N | High_Fitness_Costs | 4,03 | 0,32 |
| CCNA_03835 | 3-oxoacyl-(acyl-carrier-protein) synthase | essential | 3,95 | 0,28 |
| CCNA_00325 | hypothetical protein | Nonessential | 3,92 | 1,46 |
| CCNA_01427 | lipoprotein, SmpA/OmlA family | essential | 3,91 | 0,12 |
| CCNA_01729 | transporter, Sodium/bile acid symporter family | Nonessential | 3,77 | 0,31 |
| CCNA_02042 | trigger factor, ppiase | High_Fitness_Costs | 3,66 | 0,26 |
| CCNA_00874 | biotin synthesis protein bioC | essential | 3,62 | 1,38 |
| CCNA_00351 | primosomal protein NV' | High_Fitness_Costs | 3,53 | 0,64 |
| CCNA_02077 | acid phosphatase surE | High_Fitness_Costs | 3,32 | 1,31 |
| CCNA_00410 | acetyltransferase | Nonessential | 3,32 | 0,24 |
| CCNA_01219 | cytosolic protein | High_Fitness_Costs | 3,31 | 3,23 |
| CCNA_00024 | uroporphyrin-III C-methyltransferase/Precorrin-2 dehydrogenase/ Sirohydrochlorin ferrochelatase | Nonessential | 3,29 | 0,21 |
| CCNA_01118 | hypothetical protein | essential | 3,14 | 1,03 |
| CCNA_02315 | transcriptional regulator, Cro/C1 family | Nonessential | 3,13 | 0,32 |
| CCNA_02866 | hypothetical protein | High_Fitness_Costs | 3,11 | 0,17 |
| CCNA_00149 | transcriptional regulator, Cro/C1 family | Nonessential | 3,07 | 1,84 |
| CCNA_00425 | hypothetical protein | High_Fitness_Costs | 3,05 | 2,30 |
| CCNA_01622 | ppGpp hydrolase-synthetase relA/spoT | Nonessential | 1,60 | 0,11 |
| CCNA_01437 | transglycosylase associated protein | Nonessential | 0,33 | 0,68 |
| CCNA_02270 | transcriptional regulator, MarR family | Nonessential | 0,33 | 0,76 |
| CCNA_02309 | EF hand domain protein | Nonessential | 0,32 | 0,63 |
| CCNA_03633 | cytosolic protein | Nonessential | 0,32 | 1,10 |
| CCNA_R0078 | C starvation sRNA | NA | 0,30 | 0,91 |
| CCNA_00847 | leucine-responsive regulatory protein | Nonessential | 0,30 | 0,81 |

| | | | | |
|------------|---|--------------|------|------|
| CCNA_02148 | hypothetical protein | Nonessential | 0,29 | 1,64 |
| CCNA_03604 | hypothetical protein | Nonessential | 0,27 | 0,66 |
| CCNA_01501 | protein kinase C-like superfamily protein | Nonessential | 0,25 | 0,27 |
| CCNA_03118 | hypothetical protein | Nonessential | 0,24 | 0,84 |
| CCNA_03515 | hypothetical protein | Nonessential | 0,21 | 0,57 |
| CCNA_02942 | hypothetical protein | essential | 0,20 | 0,59 |
| CCNA_03150 | hypothetical protein | Nonessential | 0,15 | 1,20 |

Material and Methods

V. Material and methods

A. Strains and plasmid

1. Strains

| RH | Organism | Description | Reference |
|------|--------------|--|---------------------------|
| 319 | E.coli | MT607(<i>pro-82 thi-I hsdR17 (r-m+) supE44 recA56</i>) Top10 | Evinger & Agabian, 1977 |
| 129 | E.coli | MG1655 Δ <i>cyaA::frt</i> | |
| 50 | C.crescentus | NA1000 | Casadaban & Cohen, 1980 |
| 1752 | C.crescentus | NA1000 <i>spoTD81G</i> | Ronneau et al., 2016 |
| 1755 | C.crescentus | NA1000 Δ <i>spoT</i> | Ronneau et al., 2016 |
| 1819 | C.crescentus | NA1000 Δ <i>ptsN</i> | Ronneau et al., 2016 |
| 339 | C.crescentus | NA1000 Δ <i>cpdR</i> | Skerker et al., 2005 |
| 323 | C.crescentus | NA1000 Δ <i>rcdA</i> | McGrath et al., 2006 |
| 315 | C.crescentus | NA1000 Δ <i>popA</i> | Duerig et al., 2009 |
| 864 | C.crescentus | NA1000 Δ <i>clpA</i> | Grünenfelder et al., 2004 |
| 2279 | C.crescentus | NA1000 Δ <i>socAB</i> Δ <i>clpX</i> | Our Lab |
| 1674 | C.crescentus | NA1000 Δ <i>socAB</i> Δ <i>clpP</i> | Our Lab |
| 866 | C.crescentus | NA1000 Δ <i>lon</i> | Wright et al., 1996 |
| 1622 | C.crescentus | NA1000 Δ <i>ptsM</i> | Ronneau et al., 2016 |
| 2017 | C.crescentus | NA1000 <i>ptsN_{H66E}</i> | Ronneau et al., 2016 |
| 2019 | C.crescentus | NA1000 <i>ptsN_{H66A}</i> | Ronneau et al., 2016 |
| 1758 | C.crescentus | NA1000 Δ <i>ptsP</i> | Ronneau et al., 2016 |
| 2093 | C.crescentus | NA1000 <i>ptsM_{H9A}</i> | This Study |
| 2092 | C.crescentus | NA1000 <i>ptsM_{H9E}</i> | This Study |
| | C.crescentus | Δ <i>ptsP</i> Δ <i>ptsM</i> | Our Lab |
| | C.crescentus | Δ <i>ptsP</i> <i>ptsM_{H9E}</i> | This Study |
| | C.crescentus | Δ <i>ptsP</i> <i>ptsM_{H9A}</i> | This Study |

2. Plasmid

| pHR | Description | Reference |
|-----|--------------------------------------|--|
| | pNPTS138 | M. R. Alley, Imperial College London (UK), unpublished |
| 786 | pNPTS138 - <i>ptsM_{H9E}</i> | |

| pHR | Description | Reference |
|-----|--|--|
| 787 | pNPTS138 - <i>ptsM</i> _{H9A} | This study |
| 318 | pKT25 | Karimova et al. (1998) PNAS 95(10): 5752-6 |
| 505 | pUT18C | Karimova et al. (1998) PNAS 95(10): 5752-6 |
| 761 | pKT25- <i>spoT</i> | Ronneau et al., 2016 |
| 693 | pKT25- <i>ptsN</i> | Ronneau et al., 2016 |
| 885 | pKT25- <i>ptsM</i> | This Study |
| 689 | pUT18C- <i>spoT</i> | Ronneau et al., 2016 |
| 704 | pUT18C- <i>ptsN</i> | Ronneau et al., 2016 |
| 790 | pUT18C- <i>ptsM</i> | R. Hallez |
| 791 | pUT18C- <i>ptsM</i> _{H9E} | This Study |
| 792 | pUT18C- <i>ptsM</i> _{H9A} | This Study |
| 792 | pUT18C- <i>ptsM</i> _{H9A} | This Study |
| 936 | pUT18C-HA- <i>spoT</i> | R. Hallez |
| 940 | pUT18C-HA- <i>spoT</i> _{ΔACT} | R. Hallez |
| 944 | pUT18C- <i>spoT</i> ₆₃₃₋₇₄₂ (ACT) | R. Hallez |

B. Growth conditions

1. *Escherichia coli*

a) Luria-Bertani media (LB)

| | Liquid LB | LB agar |
|--------------------------|-----------|---------|
| Bacto-tryptone | 10g | 10g |
| Yeast extract (Difco) | 5g | 5g |
| NaCl | 5g | 5g |
| Agar (Difco) | - | 15g |
| dH2O to | 1L | 1L |

Escherichia coli were grown in LB broth medium at 37°C with shaking (200-250RPM).

Strains are stored at -80°C. Cells are grown as mentioned higher with appropriated antibiotics, if needed, overnight. 100μl are added to 900μl of saturated bacterial culture, cells are mixed by vortexing and freeze.

2. *Caulobacter crescentus*

a) Complex media

(1) Peptone Yeast extract media (PYE) and MacConkey

| | Liquid PYE | PYE Agar | PYE Swarmer Agar (0.3%) | MacConkey agar |
|--|------------|----------|----------------------------|----------------|
| Bacto-tryptone | 2g | 2g | 2g | 40g |
| MacConkey agar base (Difco) | | | | |
| Yeast extract | 1g | 1g | 1g | |
| 0.5 M MgSO₄ | 2ml | 2ml | 2ml | 1L |
| 0.5 M CaCl₂ | 1ml | 1ml | 1ml | |
| Agar (Difco) | - | 15g | 3g | |
| dH₂O to | 1L | 1L | 1L | |

b) Synthetic Media

(1) M2G, P2G, M2

| | M2G | P2G | M2 |
|---------------------------------|-------|-------|-------|
| 20x M2 salts | 50 ml | - | 50 ml |
| 20x phosphate buffer | - | 50 ml | - |
| 20% Glucose | 10 ml | 10 ml | - |
| 0.5 M MgSO₄ | 1 ml | 1 ml | 1 ml |
| 10 mM FeSO₄ | 1 ml | 1 ml | 1 ml |
| 0.5 M CaCl₂ | 1 ml | 1 ml | 1 ml |
| dH₂O to | 1L | 1L | 1L |

Caulobacter crescentus are grown in complex PYE media, or synthetic M2G, P2G, M2 media, at 30°C with shaking (200-250RPM)

With 20x M2 salts and 20X Phosphate Buffer

| | Components | Quantity |
|---------------------|----------------------------------|-----------------|
| 20x M2 salts | Na ₂ HPO ₄ | 34.8 g (245 mM) |
| | KH ₂ PO ₄ | 21.2 g (155mM) |
| | NH ₄ Cl | 10 g (187 mM) |

| | | |
|------------------------------|----------------------------------|-----------------|
| | dH ₂ O to | 0.5 L |
| 20X Phosphate buffer (0.4 M) | Na ₂ HPO ₄ | 34.8 g (245 mM) |
| | KH ₂ PO ₄ | 21.2 g (156 mM) |
| | dH ₂ O | 1L |

C. Solution and Buffers

| | Components | Quantity |
|--------------------------|--|-----------------|
| PBS 10X | PBS Tween 0,05% | 1L |
| | Milk (powder) | 50g |
| | K ₂ HPO ₄ 20mM | 2,72g |
| | Na ₂ HPO ₄ 100mM | 14,2g |
| | KCl 20mM | 1,5g |
| | NaCl 1,4M | 81,2g |
| SDS-Loading buffer 5X | 1M Tris (pH 6.8) | 12,5 mL (250mM) |
| | 100% Glycerol | 25mL (50%) |
| | SDS | 5g (10%) |
| | Bromophenol blue | Few grains |
| | β-mercaptoethanol | Up to 50 mL |
| SDS-Running buffer (10%) | SDS | 10 g |
| | dH ₂ O to | 0,1L |
| Z buffer | Na ₂ HPO ₄ | 8,64g |
| | NaH ₂ PO ₄ | 4,8g |
| | KCl | 0,75g |
| | MgSO ₄ ·7H ₂ O | 0,246g |
| | dH ₂ O to | 1L |

| | | |
|--|--------------------------------------|--------------|
| ONPG Substrate | ONPG | 40mg |
| | Z buffer | 10 ml |
| Na₂CO₃ 1M | Na ₂ CO ₃ | 21,2g |
| | dH ₂ O to | 0,2L |
| FACS Staining Buffer (pH 7.2) | 1M Tris | 5ml (10mM) |
| | 0.5 M EDTA | 1ml (1mM) |
| | 1M NaCitrate | 25ml (50mM) |
| | TritonX-100 | 50µl (0.01%) |
| | dH ₂ O to | 0,5L |
| | Filter sterilize | |
| Solution TSS | LB | 93 ml |
| | PEG | 10g |
| | MgSO ₄ 1 M | 1 ml |
| | MgCl ₂ 1 M | 1ml |
| | DMSO | 5ml |
| | | |
| 0,5M CaCl₂ | CaCl ₂ | 5,55g |
| | dH ₂ O to | 0,1L |
| 0,5M MgSO₄ | MgSO ₄ .7H ₂ O | 12,3g |
| | dH ₂ O to | 0,1L |

D. Preparation of antibiotics and sugars

| Antibiotic/ Sugar | Dissoluti on solution | Stock solution (µg/ µL) | Liquid LB Concentrati on (µg/ml) | Solid LB concentrati on (µg/ml) | Liquid PYE concentrati on (µg/ml) | Solid PYE concentrati on (µg/ml) |
|-----------------------------|-----------------------------|-------------------------------|--|---------------------------------------|---|--|
| Ampicilline | H ₂ O | 100 | 50 | 100 | 5 | 50 |
| Chloramphenic ol | EtOH 100% | 30 | 20 | 30 | 1 | 2 |
| Kanamycine | H ₂ O | 50 | 30 | 50 | 5 | 20 |

| | | | | | | |
|--------------------------|-------------------|------|------|------|-----|----|
| Acide Nalidixique | NaOH 0,2M | 20 | 15 | 30 | 15 | 20 |
| Oxytetracyclin | dH ₂ O | 12,5 | 12,5 | 12,5 | 2,5 | 5 |
| Spectinomycin | dH ₂ O | 100 | 100 | 100 | 25 | 50 |
| Streptomycin | dH ₂ O | 100 | 50 | 100 | 5 | 5 |
| Maltose | dH ₂ O | 20% | - | - | | |
| Sucrose | dH ₂ O | 30% | - | - | | |
| IPTG | dH ₂ O | 1M | - | - | | |
| 100% Glycerol | dH ₂ O | 100% | | | | |

E. List of the primary and secondary antibodies

| Name | Description | Reference |
|--------------------------------|---|------------------|
| Anti-SpoT | Polyclonal Anti-SpoT immunoglobulin produced in Rabbit | R.Hallez |
| Anti-MreB | Polyclonal Anti-MreB immunoglobulin produced in Rabbit | R.Hallez |
| Anti-EIIA^{Ntr} | Polyclonal Anti-EIIA ^{Ntr} immunoglobulin produced in Rabbit | R.Hallez |
| Anti-CtrA | Polyclonal Anti-CtrA immunoglobulin produced in Rabbit | R.Hallez |
| Anti-Rabbit | Polyclonal Swine Anti-Rabbit Immunoglobulins/ HRP | Firm: Dako P0217 |

F. List of primers

| oRH | sequence | Reference |
|------------|----------------------|-----------|
| 100 | gttttcccagtcacgacg | R.Hallez |
| 104 | cgctaccaagtgccgacgaa | R.Hallez |
| 174 | ggaaacagctatgaccatg | R.Hallez |

G. Bacterial genetics

1. PCR

a) DNA amplification for screening

| Mix | Program | |
|---------------------------------------|----------------|-----|
| 21,2 µL H ₂ O | 98°C – 5' | 30X |
| 6 µL Green Taq polymerase Buffer (5x) | 98°C – 30" | |
| 1,5 µL DNTPs (5mM) | 52-66°C* – 30" | |
| 0,6 µL primer 1 (20 µM) | 72°C – Kb x 1' | |
| 0,6 µL primer 2 (20 µM) | 72°C – 10' | |
| 0,2 µL Taq polymerase (1U) | | |

* Dependent of the melting temperature of the primers.

Kb = the length, in kb, of the fragment to amplify.

b) DNA amplification for cloning

| Mix | Program | |
|---|----------------|-----|
| 20 µL H ₂ O | 98°C – 5' | 30X |
| 25 µL PrimeStar GC Buffer (2x) | 98°C – 10" | |
| 2 µL DNTPs (200µM) | 52-68°C – 5" | |
| ~1µg DNA | 72°C – Kb x 1' | |
| 0,75 µL primer 1 (20µM) | or | 30X |
| 0,75 µL primer 2 (20µM) | 98°C – 10" | |
| 0,5 µL PrimeStar DNA Polymerase (1,25U) | 68°C – Kb x 1' | |

Kb = the length, in kb, of the fragment to amplify.

2. Cloning method

• Restriction

| Insert | Matrix |
|---------------------------------|---------------------------------|
| 0.5 µl of restriction enzyme 1 | 0.5 µl of restriction enzyme 1 |
| 0.5 µl of restriction enzyme 2 | 0.5 µl of restriction enzyme 2 |
| 3 µl of appropriated buffer 10X | 3 µl of appropriated buffer 10X |
| X µl of PCR product (~1 µg) | Y µl of PCR product (~1 µg) |

Final volume = 30 µl avec dH₂O

Final volume = 30 µl avec dH₂O

- Incubate for 30' at 37°C in water bath.
- Purification of restriction products.
 - Run the restriction products on agarose gel 1%.
 - Purify the restricted insert and matrix using the QIAquick Gel Extraction kit of Qiagen.
 - Resuspend purified products in 30µl of mQ water
- Ligation of matrix and insert
 - Formula used to quantify the correct amount of insert/matrix.
 - $\text{Insert (ng)} = (\text{Matrix (ng)} \times \text{Insert size (pb)} \times 3) / \text{Matrix size (pb)}$
 - Optimal ratio between the insert and the matrix should be around 3:1

| ligation |
|--|
| X µl of purified and restricted matrix |
| Y µl of purified and restricted insert |
| 1 µl T4 DNA ligase (Invitrogen) |
| 3-4 µl T4 buffer (5X) |
| Z µl mQ water |
| 15-20 µl final |

- Incubate overnight at RT
- Check results by electrophoresis on 1% agarose gel
 - Gel migration of the DNA samples on agarose gel (agarose 1%, 99%TAE 1x supplemented with 1µl ethidium bromide for 10 ml)
 - Reference marker: GeneRuler™ 1 kb DNA Ladder.

3. Preparation of plasmidic DNA

- Use the plasmid DNA extraction kit from Qiagen

4. Biparental mating

- Inoculate a fresh colony of the donor strain, *E. coli* S17-1 harbouring the plasmid to transfer, in 5 ml of LB medium supplemented with the appropriated antibiotics, at 37°C on shaker incubator, overnight.
- Inoculate a fresh colony of the recipient strain (*C. crescentus*) in PYE medium, and grow overnight at 30°C.
- Mix 50µl of *E.coli* S17-1 harbouring the plasmid to transfer, with 950µl of the *C. crescentus* of interest.
- Centrifuge mixed culture at 8000RPM for 2' and discard supernatant.
- Resuspend the pellet in 50 µl PYE and spot the resuspended culture on PYE agar.
- Incubate the plates at 30°C for 4 hours (replicative plasmid) or o/n (non replicative plasmid).
- Resuspend the spot in 5 ml PYE and plate 100 µl of the resuspended culture on PYE agar plates supplemented with nalidixic acid and appropriated antibiotics.

5. Triparental mating

- Inoculate a fresh colony of the donor *E. coli* strain harbouring the plasmid of interest, in 5 ml of LB medium supplemented with appropriated antibiotics, at 37°C on shaker incubator, overnight. This strain is deficient for conjugaison.
- Inoculate a fresh colony of the donor *E. coli* Helper strain, in 5 ml of LB medium on shaker incubator, overnight.
- Inoculate a fresh colony of the recipient strain (*C. crescentus*) in PYE medium, and grow overnight at 30°C.
- Mix 50µl of *E.coli* Helper strain, 50µl of donor *E. coli* strain harbouring the plasmid to transfer, with 900µl of the *C. crescentus* recipient strain.
- Centrifuge mixed culture at 8000RPM for 2' and discard supernatant.
- Resuspend the pellet in 1 ml of PYE medium and wash the pellet by centrifuge at 8000RPM for 2'.
- Remove 950µl of supernatant and resuspend the pellet in the 50µl left. Spot the resuspended culture on PYE agar.

- Incubate the plates at 30°C for 4 hours (replicative plasmid) or o/n (non replicative plasmid).
- Resuspend the spot in 5 ml PYE and plate 100 μ l of the resuspended culture on PYE agar plates supplemented with nalidixic acid and appropriated antibiotics.

6. Transformation bactérienne par TSS

- Inoculate RH10 (S17-1) in 5 ml of LB medium overnight at 37°C
- Dilute 10 μ l of the overnight culture in 10ml LB and incubate at 37°C until reaching a $OD_{600} \sim 0,4$.
- Centrifuge culture at 5000 RPM (2600 x g) at 4°C during 5 minutes to harvest cells.
- Resuspend cells 1ml of pre chilled TSS at 4°C and keep it on ice during 10'.
- Add 2 μ l of DNA (plasmid) to 100 μ l of cells in an eppendorf 1,5 ml.
- Keep the tube on ice during 5' to 60'.
- Add 1ml of LB and incubate at 37°C during 60' on shaker (200-250RPM).
- Plate 100 μ l 900 μ l of the samples on selective media (LB supplemented with adequate antibiotics)
- Incubate overnight at 37°C without shaking

H. Bacterial physiology

1. HPLC based (p)ppGpp quantification

2. Cell harvesting

- Incubate *C. crescentus* cells overnight in PYE at 37°C in shaker incubator.
- Dilute 1 ml of culture in 20 ml in M2G in shaker incubator.
- Dilute cells in PYE, M2G and P2G during 5h in order to reach a mid log exponential phase $OD_{660} \sim 0,3$.
- Harvest 20 ml of cells by 20 ml of culture in ice-cold formic acid to reach a final 1M concentration.
- Snap freeze the samples in liquid nitrogen and store samples at -80°C until extraction.

- The three upper steps have to be realised ideally in less than 30'.

3. Nucleotides extraction

- Quickly thaw the samples in a 37°C water bath (Frequently vortex samples to keep an homogenous temperature as lowest as possible to avoid chemical degradation).
- Keep samples for 30' with occasionally vortexing
- Centrifuge samples at 5000G for 10' at 4°C
- Decant supernatant directly onto syringe with 0.2 µm filter, filtrate

4. Concentration of nucleotides on FPLC

- At 4°C:
 - Equilibrate column with deionised (mQ) water (4ml/min)
 - Dilute samples 20x in mQ water
 - Load diluted sample onto QSepharose FF column matrix (GE Healthcare # 17051001) (4ml/min)
 - Wash column with mQ water for 3 minutes (4ml/min)
 - Elute nucleotides with 2 M LiCl, 25 mM Tris pH 8 (1ml/min)
 - Follow elution at 254nm and start collection when the peak appears
 - Wash column with 2 M LiCl, 25 mM Tris pH 8
 - The column was equilibrate with mQ water

5. Precipitation

- Four volumes of ice-cold 96% EtOH and 4 µL of 1 M KH₂PO₄ (co-precipitate) were added to the samples followed by vortexing for 2-3 seconds.
- Samples were transferred at -20°C for overnight precipitation.
- Samples were centrifuged at 5,525 G during 20 min at 4 °C.
- Pellets were rinsed with ice-cold 70% ethanol.
- Centrifuge again at 5,525 G during 20 min at 4 °C.
- Dry pellets by speed vac at 4°C.
- Resuspend pellets in ice-cold 200 µl mQ water.

- Transfer samples to 1,5 eppendorf tubes.
- Centrifuge 30' at max speed at 4°C
- Transfer supernatant in a new cold 1,5 tube
- Store samples at -20 °C until HPLC analysis

6. Strong Anion Exchange HPLC

- Nucleotides concentration was measure using Prominence-*i* LC-2030C HPLC
- Gradient program
 - Buffer A: 0.05M NH₄H₂PO₄ pH 3.4
 - Buffer B: 0.5M NH₄H₂PO₄ pH 3.4
 - Program

| Time | % of buffer B | Flow Rate (ml/min) |
|--------|---------------|--------------------|
| 0 min | 0 | 1 |
| 30 min | 100 | 1 |
| 45 min | 100 | 1 |

- Detector
 - The abundance of nucleotides were measured every 300 mlli seconds during 50' at a wavelength of 254nm

7. Motility assay

- Strains were inoculated overnight in 5ml PYE at 30°C.
- Dip the tip of a toothpick into the culture.
- Prick the toothpick in the semi-solid PYE swarmer agar.
- Incubate for 2 or 3 days at 30°C without shaking
- The motility of the strain was measured by the area of the swarm colonies.
- The motility was then normalised to the WT *C. crescentus* strain to express the relative motility of the candidates.

8. Growth curves (Bioscreen assay)

- *C. crescentus* strains are inoculated in 5 ml PYE and incubated at 30°C overnight
- The overnight culture was diluted in 2 ml fresh PYE medium to reach a final $OD_{660} \sim 0,03$, using the following formula: $x (\mu l) = 0.03 / OD_{660}$ (o/n culture)
- 200 μl of diluted culture were transferred in the wells of a 96 wells plate.
- Use the first and the last line and column as blank (PYE medium).
- The strains were grown during 24h at 30°C with measurement of density at OD_{660} taken every 8 minutes with continuous shaking.

9. Synchronization

- Incubate *C.crescentus* strain overnight in 5ml PYE medium at 30°C on shaker incubator.
- Transfer 100 μl of the overnight culture into 20 ml M2G medium and grow the cells at 30°C on shaker incubator overnight.
- Dilute the culture into 1,2L M2G until reaching $OD_{660} \sim 0,6-0,9$.
- From this step, keep cells on ice during the entire synchronization and precool all glassware.
- Harvest the cell culture by centrifuging at 6000 RPM during 10' at 4°C. and discard the supernatant.
- Completely resuspend the pellet in 5 ml ice cold PO_4^{3-} buffer with a glass pipette.
- Transfer the resuspended pellet into a 250 ml cylinder and add ice cold PO_4^{3-} buffer until 160 ml.
- Add 80 ml of ice-cold Ludox (Sigma; Ref - 420808) using a glass pipette. Seal the cylinder with parafilm and mix the cell/Ludox suspension by inverting up and down.
- Transfer cell/Ludox suspension in 8 pre-chilled 30 ml corex tubes.
- Centrifuge at 9000RPM for 35' at 4°C with deceleration at "slow".

- After centrifugation you should see two bands: The upper band representing stalked and pre-divisioinnal fraction, and a lower band containing the swarmer fraction
- With a pipette connected to a vacuum pump, remove the upper band and Ludox-buffer until reaching the lower band.
- Collect and combine two of the lower bands of each tube, with a pipette, in a new 30 ml corex tube.
- Fill the new corex tubes containing the swarmer fraction with ice cold PO_4^{3-} buffer, and mix the fraction several times by up and down.
- Centrifuge at 8000RPM for 10', at 4°C, and with "fast" deceleration.
- Repeat the two previous steps to obtain a firm pellet, but with a 5 minutes centrifugation step.
- Aspirate the supernatant with a glass pipette connected to a vacuum pump.
- Resuspend the pellet in M2G at room temperature to a final $\text{OD}^{660} \sim 0,3$
- Start the cell cycle by putting the culture at 30°C in shaking water bath. This correspond to time 0.
- Sample 4ml of culture every 20' during 140' and make a protein extract to perform a western-blot.

10. Flow cytometry (FACS)

- Fixation of cells
 - Inoculate a fresh *C. crescentus* strain of interest in 5 ml PYE on shaker incubator, at 30°C, overnight.
 - Transfer 250 μl of overnight culture into 5 ml of fresh PYE medium, and grow at 30°C until $\text{OD}^{660} \sim 0,4$.
 - Transfer 1 ml of culture in 9 ml of 77% ethanol. Mix well.
- Staining of cells
 - Wash 2 ml of fixed cells in 1 ml FACS staining buffer.

- Harvest cells by centrifugation at 8000RPM for 2',
 - Resuspend pellet in 0.1 mg/ml RNaseA diluted in 1ml FACS staining buffer. Incubate at RT for 30'.
 - Centrifuge cells at 8000RPM for 2' and remove supernatant.
 - Resuspend the pellet in 1 ml FACS Staining Buffer containing 0.5 μ M Sytox Green (Invitrogen Ref : S7020) and incubate at RT in the dark for 5 min.
 - Analyse samples using the FACS SCAN using the argon laser, with an excitation 488nm.
- Detection parameters of the FACS SCAN

| Detecteur | Voltage | AmpGain | Mode | Gate |
|-----------------|---------|---------|------|---------|
| FSC | E01 | 1.00 | LOG | - |
| SSC | 631 | 1.00 | LOG | - |
| FL1 (Alexa 488) | 613 | 1.00 | Lin | 160-640 |

I. Molecular biology

1. Purification of *C. crescentus* genomic DNA

- Inoculate a fresh culture of the strain of interest in 5 ml of PYE medium, and grow overnight at 30°C.
- Transfer 1,5 ml of overnight culture in a 1,5 Eppendorf tube and centrifuge at 12.000RPM for 10'.
- Resuspend the pellet in 567 μ l TENa, 30 μ l 10% SDS and 3 μ l 20 mg/ml proteinase K
- Mix gently and by inverting the tube up and down 4 to 6 times.
- Incubate during 1h at 30°C.
- 600 μ l of Phenol:Chloroform:Isoamyl Alcohol (24:24:1) were added to the culture and mix thoroughly.
- Centrifuge at maximum speed during 20' at 4°C.

- Transfer the liquid upper fraction of the tube in a new 1,5 ml Eppendorf tube and add 1 ml of 100% ice-cold ethanol mix thoroughly.
- Centrifuge at maximum speed during 10' at 4°C.
- Wash pellet in 1 ml ice-cold 70% ethanol.
- Centrifuge at maximum speed during 10' at 4°C. Let pellet dry at RT.
- Resuspend pellet in 100µl H₂O. And incubate at 37°C during 1h.
- Resuspend again the solution.
- Check the gDNA prep on agarose gel, and quantify the gDNA with the nanodrop.

2. Western Blot

- Protein crude extract
 - Incubate overnight a fresh colony of the strain of interest into 5 ml PYE, at 30°C, on shaker incubator
 - Dilute overnight culture in adequate medium (PYE, M2G, P2G, M2) and grow at 30°C temperature until OD₆₆₀ ~ 0,3-0,5.
 - Pellet 2 ml to 4 ml of culture (or synchronisation culture) at 10000RPM for 2'. Discard supernatant.
 - Resuspend pellet in a volume of SDS-loading buffer equivalent to OD₆₆₀ x 100 (50 if low amount of protein) of 1X SDS-loading buffer.
 - Boil samples during 10' and store at -20.
- Gel running
 - Load 20 µl of protein crude extract into a 12% acrylamide SDS-PAGE gel from Mini-PROTEAN® TGX™ Precast Gel
 - Perform a 20' to 45' migration in the Mini-PROTEAN® Tetra Vertical Electrophoresis Cell, filled with 1x SDS-Running buffer
 - Set up the current on 45 mA/gel (200V).

- Transfer
 - Use the Trans-Blot[®] Turbo[™] Mini Nitrocellulose Transfer Packs (#1704158) protocol to assemble the sandwich, comprising the SDS-page acrylamide gel, the membrane and the wattman.
 - Insert the Semi dry transfer Biorad in the Trans-Blot[®] Turbo[™] Transfer System (#1704150)
 - Run at constant voltage 14V; 3 mA/cm² (105 mA/membrane for small Biorad gel).
 - To block membrane, incubate the membrane in PBS with 0.05% Tween 20 and 5% Non-fat milk (PBS_T_M) in cold room over night.
- Immunoblot
 - Discard blocking solution and add primary antibody diluted in 5 ml of PBS_T_M. Incubate during 3 h in shaker at room temperature (RT).
 - Wash membrane 3 time with PBS with 0.05% Tween for 3 minutes each.
 - Add secondary antibody diluted in 5 ml of PBS_T_M and incubate for 1 h.
 - Wash membrane 5 time with PBS with 0.05% Tween for 3 minutes each.
- Detection
 - Mix both solutions from the Clarity[™] Western ECL Substrate, 500 ml (#1705061) Bio Rad with a ratio 1:1 (500µl solution A and 500µl solution B for 1 membrane)
 - Remove secondary antibody solution and incubate the membrane for 2 minutes at RT. Reveal the signal.

3. Bacterial two hybrid (BTH)

- Clone genes of interest in pKT25, pUT18 and pUT18C plasmids with bait and prey proteins of interest (see cloning procedure).
- Transform recombinants plasmids, pKT25, pUT18 and pUT18C, in *E. coli* MG1655 Δ cyaA::frt strain TSS competent strain (see TSS transformation procedure)

- Select transformants on Amp/Kan LB agar plates.
- Incubate double transformants with recombinants plasmids (pKT25-prey / pUT18-bait or pKT25-prey / pUT18C-bait) in LB supplement with Amp/Kan. Incubate at 30°C overnight on shaker incubator.
- Spot 2µl of the overnight culture on MacConkey agar medium supplemented with 1% maltose, Amp, Kan and 0,5mM IPTG.
- Incubate at 30 °C until there is a color difference between positive and negative controls (usually 2-3 days).

4. β-galactosidase assay

- Transform recombinants plasmids, pKT25, pUT18 and pUT18C, in *E. coli* MG1655 ΔcyaA::frt strain TSS competent strain (see TSS transformation procedure)
- Incubate, overnight, a fresh colony of interest in 200µl LB medium supplemented with appropriate antibiotics, in 24 wells plate.
- Dilute cultures and incubate until reaching OD⁶⁰⁰~0,3.
- Add 50µl of chloroform to 200µl of culture into a fresh 2 ml eppendorf tube and vortex 10".
- Add 600µl Z Buffer and mix gently.
- Add 200µl ONPG [4 mg/ml] diluted in Z Buffer, vortex 5" and incubate at 30°C until a medium-yellow appears in the tube. Time of reaction vary between 3' and 15'.
- Stop the reaction by adding 500µl 1 M Na₂CO₃.
- Centrifuge at max speed for 2' to pellet cells debris, and transfer supernatant into new spectrometry cuvette.
- Determine DO⁴²⁰ (use H₂O as blank), DO⁴²⁰ should be between 0.2 and 1.0.
- Calculate Miller Units with the following formula:
 - Miller Units (M.U.) = $DO^{420} \times 1000 / DO^{420} \times t \times v$
 - where t : reaction time (min)
 - v : volume of culture used in assay (ml)

- * : DO⁶⁰⁰ for *E. coli*
- The average of 3 replicates was taken for each condition and normalized to the WT.

5. Tn-seq

- Transposition experiment
 - Insertion of the plasmid carrying the transposase gene by biparental mating.
 - Culture in LB overnight the *E. coli* S17 strain, harbouring a plasmid carrying the *tn5* gene coding for a hyperactive transposase Tn5. This gene is under the control of the xylose promoter. The plasmid also presents a kanamycin resistance cassette (adapted from christen et al 2011).
 - Culture in PYE overnight the *C. crescentus* strain of interest.
 - Perform a biparental mating as mentioned higher in "biparental mating" section.
 - Preliminary tests allowed us to determine the number of matings required to reach an efficiency of transposition of 10.000 clones per plates.
 - For the Tn-seq experiment, an average of 300.000 clones per condition was determine as a correct number of transposition events to disrupt the whole genome of *C. crescentus*.
 - The suppressor screen was performed with the same principle on approximately 200.000 clones
- Tn-seq analyse
 - The 300.000 clones mentioned higher were collected and resuspended in PYE.
 - A genome extraction was then performed on 50µl of resuspended culture for each condition tested.
 - Illumina single reads sequencing was performed to identify every transposon insertion sites.
 - A qualitative analysis of the results was performed using the FasQC software (Andrews, 2010)

- A truncation of 5 nucleotides at the 5' and 3' of each reads was performed. This was done to avoid quality defaults due to insertions in the end or the beginning of the genes.
- All the reads were then mapped on the genome of NA1000 *C. crescentus* using the Burrows-Wheeler Aligner algorithm (Li & Durbin, 2010), and the coordinate of the transposon insertion were determined thanks to the previous step.
- The reads were ordered following the coordinate of insertion. The insertions at a similar locus were added to map the number of transposons on the genome base per base.
- A sum of the reads for each gene was perform to determine the number of transposon insertion per gene. This was done on the full length genes but also on the 80% inner region of the genes. This was done because essential genes have been shown to be still functional when disrupted in the beginning and the end of the coding sequence of this gene. The removal of 10% of the gene coding sequence in our analyse allowed us to draw more accurate conclusions.
- Finally, due to the sensitivity of the approach, we decided to set a lower threshold for the acceptance of a single insertion. This lower threshold was set as a minimum of 5 reads to consider a single insertion at a locus. This allowed us to reduce the background noise.
- Analyse of results
 - To analyse the results obtained, we compared the insertions in the WT NA1000 *C. crescentus* strain with the transposons insertions in the strains of interest.
 - First, the number of reads per genes were normalized by calculating the ratio between the number of total reads from the reference strain and the strain of interest. Reads for each gene was then normalized by multiplying the gene transposon insertion by this ratio. This avoid irreverent difference between the strains to compared due to experimental differences in the number of reads.
 - The number of reads per gene of the strain of interest was deleted by the number of reads of the WT strain. This was done to eliminate extreme ratios caused by very low amount of transposons insertion in each strains. A Δ of 200 was taken as threshold.

- The ratio of reads between the the WT condition with the strain of interest were then determine to be able to compare the two conditions.
- Finally, the genes harbouring a 3-fold enrichment or impoverishment were listed as potential candidates. This list was then analyzed to connect the candidate to the condition studied.

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VI. References

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